UPTAKE AND SEDIMENTATION OF ARSENIC, NICKEL AND URANIUM FROM URANIUM MINE-IMPACTED WATER BY *CHLAMYDOMONAS NOCTIGAMA*

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ABSTRACT

The primary aim of the research summarized in this thesis was to confirm or refute that algae are involved in metal sedimentation from surface water, and whether this activity, if any, is enhanced by increased phosphorus availability.

A small-scale laboratory-based experiment was devised to elucidate the role of the chlorophyte alga *Chlamydomonas noctigama* in the removal of arsenic, nickel and uranium from mine water. Results indicated that the presence of *C. noctigama* significantly enhanced the removal of these metals relative to treatments without cells. Metals were present in greater concentrations in particulate matter in treatments with cells compared to treatments without cells, and there was a corresponding decrease in the concentrations of dissolved metals. This leads to the conclusion that sedimentation was mainly biotically induced.

Additional evidence of biotic involvement in metal removal from water by *C. noctigama* was obtained by using EDX spectroscopy and X-PEEM spectromicroscopy to observe complexation of arsenic, nickel and uranium to *C. noctigama* cells. Arsenic, the metal which was present at the lowest concentration in the DJX water, was present on scanned cells in low concentrations, and nickel and uranium, which were present at high concentrations in the DJX water, were present at higher concentrations. Examination of a single cell using X-PEEM spectromicroscopy showed uranium co-localized with carbon and phosphorus on the exterior of the cell.

Crystalline particulate matter may have increased in the presence of cells. EDX spectroscopy showed that the crystalline particulate matter was possibly hydroxyapatite that contained various metals, including arsenic, nickel and uranium. EDX spectroscopy was used to determine the frequencies at which the cell-metal and particulate matter-metal associations occurred, and the relative concentrations of the metals associated with particulate matter.

No correlation between metal removal and phosphorus concentration in the media, or between algal density and phosphorus concentration was observed. However, phosphorus concentrations were not growth-limiting in these experiments, and so the effect of phosphorus on abiotic precipitation of metals remains unclear.

Results suggest two mechanisms by which *C. noctigama* may remove arsenic, nickel and uranium from solution: by direct sorption to the exterior of the cell, and by sorption to a cell product.

An experiment using cells preserved in Lugol's iodine (a common phytoplankton sample preservative) indiated that Lugol's preserved samples could not be used to quantify metals using spectroscopy. Consequently, historical samples preserved with Lugol's iodine cannot be analyzed by this method.

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LIST OF ACRONYMS AND ABBREVIATIONS

Å	Angstrom
ANOVA	Analysis of variance
As	Arsenic
°C	degrees Celcius
DJX	Dominique-Janine Extension, an open pit mine at Cluff Lake uranium mine
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive X-ray spectroscopy
eV	electron volt
g	gram
g	gravities
h	hour
ICP-MS	Inductively-coupled plasma-mass spectroscopy
ITS	Internal transcribed spacer
keV	kiloelectron volt; 10^3 electron volts
KH_2PO_4	Monobasic potassium phosphate
L	litre
m	meter
mg	milligram; 10^{-3} grams
mL	milliliter; 10^{-3} litres
µg	microgram; 10^{-6} grams
µg	microlitre; 10^{-6} litres
µmol	micromole; 10^{-6} moles
Ni	Nickel
P	Phosphorus
PCR	Polymerase chain reaction
PGM	Plane grating monochromator
rDNA	Ribosomal deoxyribose nucleic acid
rpm	revolutions per minute

s	second
SEM	Scanning electron microscopy
SPHINX	Spectromicroscope for photoelectron imaging of nanostructures with X-rays
SRC	Saskatchewan Research Council (Saskatoon, Saskatchewan)
SRC	Synchrotron Radiation Center (Stoughton, Wisconsin)
STXM	Soft X-ray scanning transmission X-ray microscopy
TEM	Transmission electron microscopy
U	Uranium
USAC	University of Saskatchewan Algae Collection
VLS-PGM	Varied line space plane grating monochromator
XAFS	X-ray absorption fine structure
XANES	X-ray absorption near edge structure
X-PEEM	X-ray photoemission electron microscopy

CHAPTER 1. INTRODUCTION

1.1 Remediation of contaminated mine water

Uranium and metal mining and milling operations produce large volumes of water that contain a mixture of contaminants including radionuclides and heavy metals. There are two main impact scenarios. In the first, treated effluent is released into downstream environments. Such effluent release is regulated and must conform to federal and provincial surface water quality objectives (CCME 2002), and for this reason will not be considered in this thesis. In the second scenario, a flooded pit remains after ore extraction from shallow (typically less than 200 m deep) deposits. Inevitably, the pit water has elevated metal and mineral concentrations, the extent of which is dependent on the amount of contaminated waste placed in the pit, contaminant leaching from the pit walls, and surface runoff from the surrounding area. The quality of the water in pit lakes becomes a major concern at the time of mine decommissioning, when it must meet surface water quality objectives (Lee *et al.* 1993). Typically, to achieve surface water quality objectives, most of the contaminated pit water is pumped from the pit, treated chemically and released. Then the pit is refilled with uncontaminated water from a nearby lake. Such remediation is costly and leads to contamination of the downstream aquatic ecosystem, principally by an increase in salinity. Two of the uranium mining companies in Saskatchewan, Cameco Corporation and AREVA, have expressed an interest in exploring the potential of remediating contaminated pit water *in situ* by biological means with the purpose of minimizing downstream ecosystem effects.

There is some evidence that over relatively short periods of time (around 20 years) concentrations of toxic metals in the surface waters of some contaminated pits have decreased to acceptable levels through natural processes without chemical treatment. Presumably contaminants are removed from surface waters by both inorganic precipitation and the uptake of contaminants by microorganisms, primarily bacteria and

algae, that sediment out of the water column when they senesce and die. Metal-particulate matter associations are well-documented in riverine, marine and lacustrine environments and are important contributors to precipitation of heavy metals in all of these systems (Hunt 1983; Sigg *et al.* 1987; Vignati and Dominik 2003; Degueldre *et al.* 2004). Aggregates of algae and inorganic matter are well documented (Avnimelech *et al.* 1982; Oliver *et al.* 1985; Fukuda and Koike 2004), and it is not unlikely that algae are an important contributor to metal precipitation. In Saskatchewan, naturally occurring improvement in surface water quality over time has been observed at the Gunnar Pit at Uranium City (CanNorth 2004) and the D-Pit at Cluff Lake mine (AREVA 2002a and b), and it has been suggested that much of the sedimentation of metals was a result of activity by microorganisms (AREVA 2002a and b). After mining, both pits were permitted to fill with water and have remained untreated in the intervening years, since 1966 in the case of the Gunnar Pit, and since 1985 in the case of the D-Pit (Cogema Resources Inc. 2000). However, it is not possible to directly attribute all of the observed improvement in surface water quality in these pits to sedimentation processes. Both the Gunnar Pit and the D-Pit had inputs of uncontaminated fresh water that may have reduced contaminant levels by producing a distinct surface layer of clean water. The Gunnar Pit was opened to inflow from Lake Athabasca between 1964 and 1966, and in 1985 the D-Pit was flooded with spring run-off that replaced some of the pit water by clean water. A major question relating to these naturally occurring reductions of metal concentrations in surface water is how much is related to abiotic processes (e.g. introduction of clean water and chemical precipitation of contaminants), and how much is mediated by biological processes.

An obvious question about the natural processes that remove contaminants from surface waters and deposit them in the sediments is whether or not the removal is permanent. Typically, deep water bodies in temperate regions undergo a fall and spring turnover when the entire water body mixes. This occurs in the spring when the entire water column is at a uniform temperature and strong winds mix the water throughout its depth, and again in the fall when the surface water becomes cooler and denser than the bottom water so that the whole water column turns over and mixes. Such water bodies are said to be holomictic (Kalff 2002). If complete mixing of the water column occurs in

a pit lake, then the sedimentation processes may not provide a suitable remediation of the surface water. However, not all lakes mix in the manner described. Meromictic lakes do not mix completely because they form a stable, dense bottom layer (with high concentrations of total dissolved solids and low dissolved oxygen) that resists mixing with the surface water. Many flooded, mined-out pits have morphometries ideal for the development of meromixis. First, they are steep-sided and have a low surface area to depth ratio, so that the kinetic energy imparted by the wind is insufficient to mix the water completely. Second, as contaminants are transferred to the lower reaches of the water column, the density of the bottom layer of water increases, and at some point may become greater than that of water at 4 °C, so that the fall overturn will not occur, thus establishing meromixis. The Gunnar Pit at Uranium City has steep walls, a small surface area and is greater than 100 m deep, and has exhibited meromixis over a long period of time (since 1981), with a stable, anoxic monomolimnion below about 75 m. The D-Pit at the former Cluff Lake Mine is only about 25 m deep, but it has steep walls, a small surface area. It also exhibits meromixis, with the (anoxic) monomolimnion stable below 15 m. Both of these pits sustain water qualities better than surface water quality objectives, with the exception of iron in the D-Pit and iron and radium-226 in the Gunnar Pit (Cogema Resources Inc. 1991; CanNorth 2004). In cases such as these, metals that sediment to the bottom of a pit are localized to sediments and their subsequent mobility is limited by sorption to solid organic matter and mineral conjugates. This depends on the speciation of the specific elements and on the nature of the substrate to which they are bound (Kauffman *et al.* 1986; Bernhard *et al.* 1996; Moldovan *et al.* 2003; O'Day *et al.* 2004; Denecke *et al.* 2005).

1.2 Metal uptake by phytoplankton

Microbial populations, including eukaryotic unicellular algae and prokaryotic autotrophs and heterotrophs, may be the principal mediators of metal sedimentation from surface waters (Pawlik-Skowrońska and Skowroński 2001). In order to live in metal-contaminated water, microorganisms must have one or more mechanisms of tolerance. There are numerous specific instances of microbes with innate or acquired

tolerances to high contaminant metal concentrations, including algae from diverse phyla, and autotrophic and heterotrophic bacteria. These organisms are of particular interest for bioremediation activities (Knauer *et al.* 1997; Siciliano and Germida 1997; Rai and Gaur 2001; Dessouki 2005). Indeed, there is a link between the organisms' mechanism of tolerance to metals and how they sediment the metals.

Known mechanisms of algal resistance to toxic metals include passive metal ion binding on cell surfaces, precipitation of insoluble complexes on the cell surface, excretion of metabolites to detoxify metals extracellularly, use of energy-driven efflux pumps to pump metals outside of the cell, intracellular deactivation with proteins or polysaccharides, enzymatic conversion of oxidation states of the metals, methylation, and vapourization and elimination of volatile chemical species (Maeda and Sakaguchi 1990; Rai and Gaur 2001). Three types of mechanism are most likely to be relevant to metal sedimentation from contaminated water: passive sorption onto cell walls, uptake into cells, and production of metal-chelating cell products. If it were possible to determine the mechanism or mechanisms of algal tolerance, it might be possible to determine which algal species were best suited for the bioremediation of metal contaminated sites.

Passive sorption onto cell walls is the simplest mechanism. Cell walls sorb dissolved metal ions from solution in proportion to the number of proton-binding sites available on the cell surface (Kiefer *et al.* 1997; Kotrba *et al.* 1999). Cell walls are composed of a highly organized, layered lattice of crystalline glycoproteins held together by non-covalent bonds (Roberts 1974; Jiang and Barber 1975; Harris 1989; Adair and Snell 1990). Proteins and peptides embedded in cell walls serve a variety of functions, including crosslinking between elements of the glycoprotein framework (Voigt and Frank 2003), as precursors to wall components during growth, and as wall-embedded transport proteins (Hannikenne *et al.* 2005). Surface functional groups may include carboxyl, hydroxyl, phenolic and sulfhydryl groups (Kiefer *et al.* 1997; Kelly *et al.* 2001). Much work has been done to model metal and ligand interactions with cell surface functional groups and, more generally, with organic particulate matter, but the results are as inconclusive and incomplete as the reality is complicated (Morel and

Hering 1993; Schiewer and Wong 2000; Campbell *et al.* 2002; Meylan *et al.* 2003; Hudson 2005; Luoma and Rainbow 2005).

Various strategies using "immobilized" (dead) fungal and algal biomass to remediate heavy metal and radionuclide contaminated sites (both aquatic and terrestrial) have demonstrated that passive metal sorption occurs. The cellular biomass acts as a selective ion exchanger and removes various metals, including arsenic, nickel and uranium, from wastewaters and soils (Nakajima *et al.* 1981; Mahan *et al.* 1989; Leusch *et al.* 1995; Schiewer and Wong 2000; Loukidou *et al.* 2003; Akhtar *et al.* 2004). Metal removal also occurs when live algae are used as the sorbent (Coleman *et al.* 1971; Bates *et al.* 1982; Mann and Fyfe 1984; Hashemi *et al.* 1994; Knauer *et al.* 1997; Mehta and Gaur 1999; Macfie and Welbourn 2000; Campbell *et al.* 2002; Davis *et al.* 2003; Deng *et al.* 2003; Murray *et al.* 2003; Kobayashi *et al.* 2005; Kola and Wilkinson 2005). If live cells take up metal actively, the amount of metal sorbed would be greater than that sorbed by a cell that did not take up metal actively, as would certainly be the case for a dead cell. Over the duration of an experiment, live cells grow and divide, and would provide a greater net surface area, a greater net biomass and more specific binding proteins for metal sorption than would dead cells, and over a longer period of time, allowing continuous decontamination. Live cells may also up-regulate production of surface moieties that sorb metals as the metal concentrations increase, which would be very useful to a cell (Valls *et al.* 2000; Mejáre and Bülow 2001). Most studies of metal sorption by dead biomass use cells that have been modified chemically by treatment with strong acids. Presumably this treatment would expose anionic sites on the cell surface, or lyse cells to increase the surface area available for metal binding. However, it is difficult to generalize about whether metal sorption is greater by live cells or dead cells if methodologies are very different. Some differences are experiment duration, phosphorus concentration of the media, species used, and the metals measured. (Table A.1 in Appendix 1 summarizes the quantitative data from numerous researchers' work into metal sorption by numerous microbial species, and includes metal sorption by live and dead algal cells.)

Uptake of metals into cells is the second mechanism relevant to sedimentation of metals by algal cells. Algae rely on an exogenous supply of minerals, organic molecules

and trace metals for successful metabolism. There are a variety of uptake pathways and intracellular storage methods that ensure the availability of particular metabolites when they are needed (Harris 1989; Fortin and Campbell 2001; Rosakis and Köster 2005). Cells may also up-regulate production of anti-toxicants that protect them from oxidative stress produced by high metal concentrations (Howe and Merchant 1992). For instance, in the chlorophyte *Chlorella*, intracellular proline accumulation was induced by high concentrations of nickel and other metals (Wu *et al.* 1998; Mehta and Gaur 1999). Proline is produced as a general response to stress, but its precise role is not known. It may function either as an anti-oxidant to prevent lipid peroxidation (and subsequent membrane destruction) or it may be involved in metal sequestration in the cell (Mehta and Gaur 1999; Siripornadulsil *et al.* 2002). Another mechanism of intracellular accumulation occurs in conditions of abundant phosphorus when some algae, including algae of the genus *Chlamydomonas*, take up and sequester phosphorus in polyphosphate bodies (Yu and Wang 2004; Eixler *et al.* 2005). Cationic metals taken up by the cell are taken into the polyanionic bodies and are sequestered there for the duration of the polyphosphate storage. Production of polyphosphate bodies may be increased upon stimulation by some metals, resulting in an increased intracellular metal concentration (Hashemi *et al.* 1994), and may serve a protective function against metal toxicity (Twiss and Nalewajko 1992; Kobayashi *et al.* 2005). If a metal has a similar coordination chemistry to phosphate, as is the case with arsenic oxidized to arsenate, then increased uptake of the metal may occur by virtue of the similarity (Hellweger *et al.* 2003). To understand metal uptake for the purpose of remediation of contaminated water bodies, the particular mechanism is not relevant, but it is important to know if metals are taken into the cell actively. If so, they may be localized in organelles or polyphosphate bodies within the cell.

In the third mechanism, cells may export metal-chelating products such as phytochelatins and siderophores. Phytochelatin and siderophore production is a common strategy for metal-scavenging used by many organisms (Ahner and Morel 1995; Dreschel and Jung 1998; Schmidt 1999; Rai and Gaur 2001). Phytochelatin and siderophore production can be stimulated by the presence of a variety of metals in the media (Ahner and Morel 1995; Leal *et al.* 1999; Neu 2000; Mejáre and Bülow 2001;

Renshaw *et al.* 2003; Gupta *et al.* 2004; Raab *et al.* 2004). Recall that intracellular proline concentrations are increased upon stimulation by exogenous metals; high intracellular proline stimulates the production of glutathione (GSH) (Siripornadulsil *et al.* 2002), which is a precursor to phytochelatin synthesis (Rai and Gaur 2001). In conditions of high cadmium, cells export phytochelatin and cadmium together (Lee *et al.* 1996). Croot *et al.* (2000) report the production of metal-chelating ligands by cyanobacteria, eukaryotic algae and dinoflagellates stimulated by dissolved copper, and that the rate of production of the metal-chelating ligands was dependant on the copper concentration. Metals in an outflow of acid rock drainage in Yukon Territory were removed from the water and localized in polysaccharide exudate of algal biofilms on rock (Lawrence *et al.* 1998). The nature of metal-chelating cell products in the context of sedimenting toxic metals is not well known. It is likely that these cell products and the metals sorbed to them contribute to the colloidal fraction of water, and it is likely that they would combine with other particles in the water to sediment out of surface water (Vignati and Dominik 2003; Hunt 1983; Sigg *et al.* 1987).

Another question pertaining to remediation of metals-contaminated pit-lakes is whether the process of metal removal and subsequent sedimentation from surface water by biota can be accelerated. Nutrient availability often limits algal growth (Redfield 1958; Schindler 1980), and so nutrient amendments may be very useful to encourage growth of biota, and so increase the rate of contaminant removal. Phosphorus is frequently a limiting nutrient in fresh water systems and the addition of phosphorus stimulates increased growth, which may result in proportional increases in metal uptake (Wang and Dei 2001a; Dessouki *et al.* 2005). Phytoplankton density, measured by chlorophyll *a* concentration, increased in mesocosms fertilized with potassium phosphate in the Dominique-Janine Extension (DJX) at Cluff Lake uranium mine (Figure 1.1) (Dessouki 2005; Dessouki *et al.* 2005.). There was a decrease in the surface water concentrations of several metals and a concomitant increase in the concentrations of metals in sediment traps (see below). Thus, sedimentation rates did increase as nutrient availability increased. However, the experiment could not assess the relative sedimentation rates induced by biota compared to chemical precipitation by the addition of phosphorus. Nor could it determine if the uptake by individual cells was increased by

Figure 1.1 Dominique-Janine Extension (DJX) pit at Cluff Lake uranium mine in 2003, with mesocosms. Photo: T.C.E. Dessouki.

phosphorus additions, or if the observed increase was mainly achieved because of the increased net biomass and resultant increased sedimentation of cells. Finally, phosphate loading may increase the ability of organisms to grow in the presence of high concentrations of metal (Hashemi *et al.* 1994; Wang and Dei 2001a), which may be related to increased sequestration of metals in polyphosphate bodies in conditions of high phosphorus availability.

Obviously, the full picture of sedimentation of metals by phytoplankton has only been sketched very vaguely, with many details missing or obscured by the complexity of the subject. Careful study of these physical and biological phenomena can improve our knowledge of metal sedimentation from contaminated surface waters in general and pit lakes in particular, and may facilitate the development of practical applications.

1.3 Study of sedimentation of metals by phytoplankton

1.3.1 Field and laboratory studies

Dessouki *et al.* (2005) used a mesocosm experiment at the DJX pit at Cluff Lake uranium mine and showed that as the phosphorus concentration was increased there was a significant linear-increase in the sedimentation of arsenic, cobalt, copper, manganese, nickel, radium-226, uranium and zinc. There was a concomitant significant linear decrease in surface water concentrations of all of these metals, except radium-226 and uranium (which was nearly significant; $p = 0.065$). Neither molybdenum nor selenium showed any response to phosphorus additions. The study concluded that the removal of metals from the water and sedimentation on the bottom was probably the result of increased algal growth, but without direct proof of cell-metal interactions this conclusion is tentative. Pickhardt *et al.* (2002) performed another mesocosm study of mercury biomagnification in freshwater food webs, using phosphorus amendments to stimulate algal blooms. The authors note the correlation of high phytoplankton numbers with low biomagnification but do not connect this result to the possibility that metals were removed by sedimentation in the algae; neither do authors of a subsequent paper referring to this work in a further study of the same problem (Chen and Folt 2005).

Field experimental approaches to show algal involvement in uptake and

sedimentation of metals are important because they show what happens on a large scale in a mostly non-constructed environment. However, in mesocosm-scale experiments many variables cannot be controlled and obtaining adequate data replication can be difficult. In the case of Dessouki *et al.* (2005), as noted above, the relative amount of inorganic precipitation was unknown, the role of the large bacterial populations in the mesocosms was unknown, and the algal communities were not identical in each mesocosm. In contrast, laboratory studies of metal uptake generally use axenic cell cultures and strictly control the contributing variables. Because laboratory experiments tend to be on a small-scale, a large number of replicates can be used to reduce statistical variance. To increase the validity of laboratory studies, researchers can use test solutions with concentrations of metals relevant to those found *in situ* and cell lines that are able to grow in those conditions. However, many metal uptake experiments use organisms that are not acclimated or are non-resistant to the toxic metal or metals to which they are exposed (Coleman *et al.* 1971; Rangsayatorn *et al.* 2002), which may result in an underestimation of uptake due to delays in production of metabolites necessary to resist the toxic effects of the metals (as described in 1.2), or due to cell death. Additionally, non-acclimated cell lines and non-resistant species are not likely to have any practical use *in situ*.

Many techniques and analytical methods can be used both in the field and in the lab. Most studies of metal uptake by algae have used a variety of biochemical, molecular biology and organic chemistry techniques (Pradines *et al.* 2005). In the interest of brevity I will describe only those that are particularly relevant to this study.

ICP-based techniques such as Inductively-coupled plasma-mass spectroscopy (ICP-MS) are the standard for work that requires accurate quantitative measurement of individual elements at very low concentrations. A sample can be fractionated according to size (most simply by filtration) and the concentrations of metal in each size-fraction can be measured and the relative proportions compared, for instance to compare between dissolved metals and metals sorbed to solids. Measurements of metals are direct. However, a mass of homogeneous sample is required for ICP-MS analysis, so ICP-MS does not help to determine the location of metals or the mechanisms of uptake or binding on the small scale of individual cells.

One way to determine the relative concentrations of cellular and intracellular metals is to use a metal-chelating agent (frequently ethylenediaminetetraacetic acid (EDTA)) to wash metals from the surface of the cell (Bates *et al.* 1982; Hudson and Morel 1989; Knauer *et al.* 1997; Macfie and Welbourn 2000; Hassler *et al.* 2004). The concentration of metal in the sample is measured before and after washing, and the difference between the two measurements is presumed to be the mass of metal taken up by the cell. It is an excellent technique for short-term studies exploring uptake kinetics, but is not ideal for all applications. First, intracellular metal is measured indirectly; second, none of the chelating agents remove the different sorbed metals equally because different metal species are adsorbed at different pH levels (Hassler *et al.* 2004); and third, there is a strong likelihood that an unknown (and variable) fraction of the cells would be dead and broken prior to treatment and an additional fraction subject to breakage from handling and from exposure to the chelating agent (Hassler *et al.* 2004), with the result that estimates of extracellular metal may be exaggerated. For this reason this technique would provide unreliable estimates of extracellular and intracellular metal concentrations in studies where metal uptake is allowed to occur over a period of days to weeks, when numerous cells would senesce and break.

These techniques provide useful, albeit indirect, information about the nature of cell-metal interactions and metal sedimentation. However, the best way to prove that metal removal from surface water happens by the action of cells is to view metals directly in association with cells.

1.3.2 Microscopy and synchrotron spectroscopies

If we can see metals in or on a cell, we verify that a cell-metal interaction occurs, and we gain information about the nature of that cell-metal interaction, i.e. how metal removal occurs. The determination of the subcellular localization of metals is made difficult, however, by the small size of algal cells and the low concentrations of trace metals sorbed to or taken up by the cells. Traditional microscopic and spectroscopic methods have been applied to this problem. Transmission electron microscopy (TEM) and electron dispersive X-ray spectroscopy (EDX) have been used to observe the

accumulation of extracellular uranium on the surface of chlorophyte algae (Mann and Fyfe 1985) and bacteria (Liu *et al.* 2002; Suzuki *et al.* 2005), and of various other metals in and on bacteria (Gonzalez and Jensen 1998; Jackson *et al.* 1999; Liu *et al.* 2001). EDX has also been used in conjunction with scanning electron microscopy (SEM) to map localization of extracellular metals (Schulz-Baldes and Lewin 1975). Both TEM and SEM provide excellent resolution and detail for viewing samples of small individual cells, but for viewing and photography non-conductive materials, such as cells, must be heavily coated (around 200 Å) with conductive metal. This coating interferes with the acquisition of EDX spectra that reflect the elemental composition of the sample. To avoid this problem, samples can be carbon coated for the acquisition of spectra, but as a result image quality is severely compromised, and the sample is usually destroyed by the heat of irradiation. The main benefit of EDX is that a good qualitative estimate of most of the elements in a sample can be made in a single scan. The main limitations are: EDX cannot detect very low metal concentrations, quantitative measurement of elements is difficult, and the minimum area scanned by the beam to obtain spectra is too large (around 1 μm) to be useful when looking at very small cells (only 2 to 6 μm long). We are getting closer to being able to observe the cells at the correct scale, but the sensitivity of metal detection is inadequate.

Confocal microscopy can be used to acquire three dimensional images of live cells and tissues, and the location of some elements can be seen by use of dyes and labeling probes (Zucker 2006). Most work in this field with confocal microscopy has been used to investigate cell polysaccharides and exudates of relatively large biofilms and not with individual cells, especially not with extremely small cells (Lawrence *et al.* 1998; Decho and Kawaguchi 1999; Neu *et al.* 2004). This information can then be used indirectly to enhance the understanding of the co-localization of metals and polysaccharides. Viewing metals depends on the availability of suitable probes (Wendt-Larsen *et al.* 2001), which are taken up by cells and fluoresce upon illumination. To be seen, fluorescence label concentrations must be quite high, in fact much higher than relevant trace metal concentrations. This is why confocal microscopy is not useful for the direct viewing of trace elements in very small cells in ecologically relevant situations.

Synchrotron spectroscopic techniques such as X-ray absorption fine structure (XAFS) and X-ray absorption near edge structure (XANES) promise good results for measuring the speciation of elements adsorbed to cells (Kelly *et al.* 2001; Templeton *et al.* 2001; Merroun *et al.* 2005; Denecke *et al.* 2005). However, samples must be homogenized, or the complexes of interest purified and recrystallized in quantity. Undoubtedly, such sample preparations modify the cell such that it is not a realistic representation of itself in life. What is more, these techniques do not help us to determine the location of metals or mechanisms of uptake or binding on the small scale of individual cells, and there is no way to differentiate between mineral-bound metal and that bound to organic matter.

Synchrotron spectromicroscopic techniques use very bright light at specific wavelengths to look at samples. An especially promising technique is X-ray photoelectron emission microscopy (X-PEEM), which measures electrons emitted upon irradiation of the sample across a range of energies to generate pixelated movies from which detailed elemental and speciation information can be extracted as maps and spectra. Very low concentrations of elements are detectable and cell-metal complexes have been successfully observed at a resolution of 10 nm (Frazer *et al.* 2004) for microtomed and unsectioned samples (Gilbert *et al.* 2000; Labrenz *et al.* 2000; Frazer *et al.* 2003a; Frazer *et al.* 2003b; Chan *et al.* 2004).

X-PEEM has been used to characterize cell-metal complexes in biofilms and to probe the nature of mineralized filaments associated with cells (Chan *et al.* 2004). Non-conductive matter can be coated with an ultra-thin layer (around 10 Å) of conductive metal. Drawbacks include that samples must be ultra-high vacuum compatible, the best samples are perfectly flat, and quantitative data is difficult to obtain (Hitchcock *et al.* 2002). A problem associated with working in a high vacuum is that it is difficult to work with aqueous samples (Frazer *et al.* 2004). Despite practical difficulties, it is likely that X-PEEM is an analytical technique that can be used to view cell-metal interactions on the small scale and at relevant concentrations.

A potentially interesting sample preparation method is the use of Lugol's iodine for spectromicroscopy. Lugol's iodine is used to fix and stain cells for identification and long-term storage (Lund *et al.* 1958; Woelfl and Whitton 2000). It is worth investigating

its utility as a preparation for samples for spectromicroscopy because Lugol's-preserved cells maintain excellent morphological detail. There are abundant historical samples preserved with Lugol's iodine because it is a simple, field-ready preservative. However, the utility of Lugol's iodine as a preservative for spectromicroscopy does not appear to have been studied.

In conclusion, to obtain a complete picture of metal uptake and sedimentation by algae, it is necessary to use more than one technique. Laboratory studies using carefully designed experiments can be used to deduce what is happening in a small, controlled system. A combination of analytical techniques, as described above, can be used to show changes in metal concentrations between the dissolved phase and the solid phase in the presence of algae, and to show the precise subcellular location of metals associated with cells.

1.4 Study approach and research objectives

In consideration of the above, I chose to use a small-scale laboratory experiment to elucidate the roles of algae and phosphorus in the sedimentation of metals from contaminated mine pit surface water. The experimental variables were controlled so that the responses would be attributable to the contributing variables. Several replicates were used to reduce sample variance.

One species of algae was used in this study, to prevent potential problems from different communities in different mesocosms and species composition shifts that may have occurred as a result of competition or metal toxicity. The experimental organism (Figure 1.2) used was a chlorophyte alga, determined to be *Chlamydomonas noctigama* Korshikov based on DNA sequence similarity. *C. noctigama* USAC 223 was isolated from a sample of water taken from the DJX pit in September, 2004. After culturing the alga to an axenic state, genomic DNA was isolated, and a 1200 base pair segment corresponding to the 3' end of the 26S rDNA, the 5.8S rDNA, and ITS1 and 2 was amplified by PCR (5' primer CTTAGTTGGTGGGTTGCC; 3' primer TCCTCCGCTTA TTGATATGC). The amplified DNA fragment was sequenced from each end and compared to available database sequences (NCBI 2006). The DJX isolate sequence was

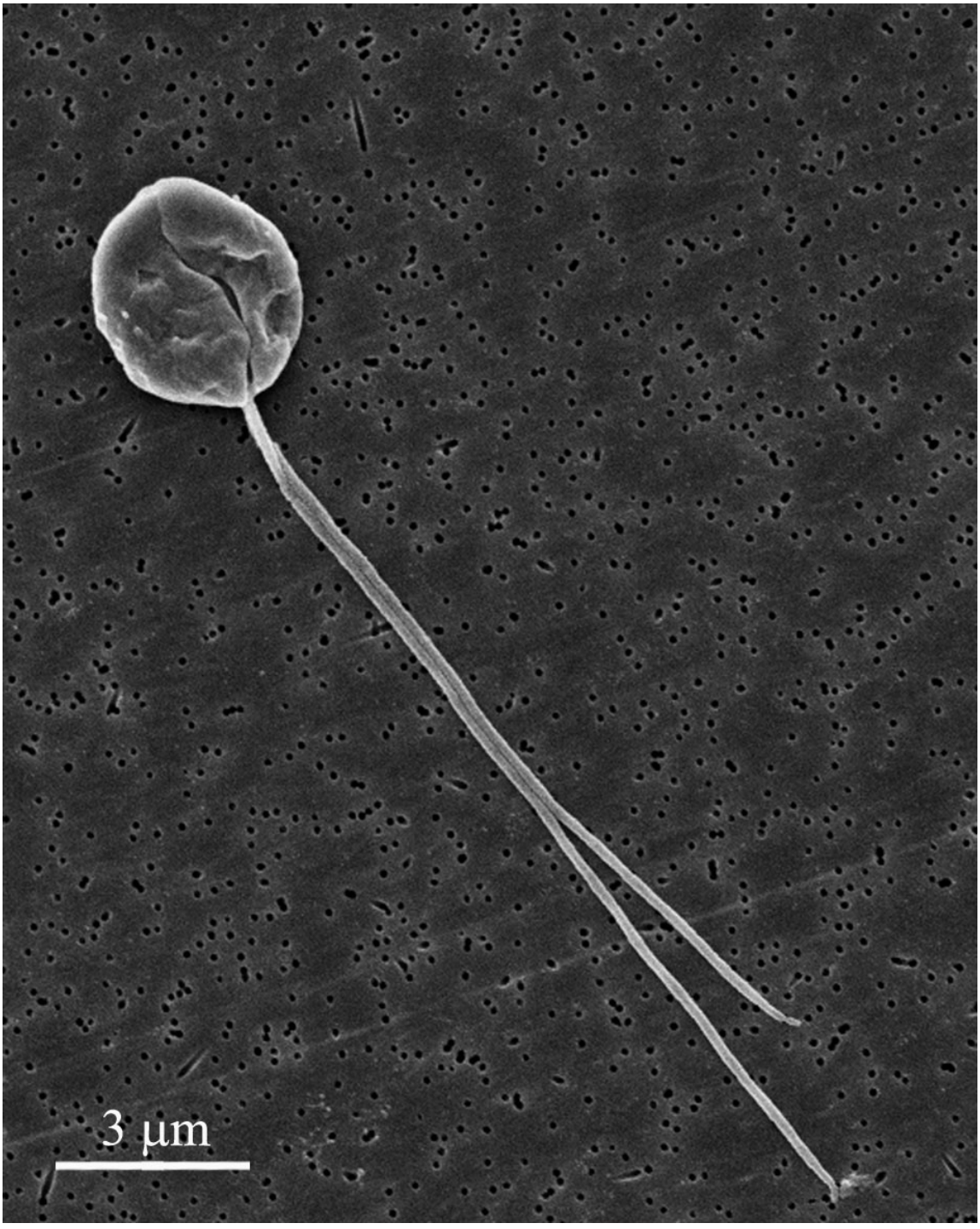


Figure 1.2 Scanning electron micrograph of a *Chlamydomonas noctigama* cell from DJX pit.

also directly compared to the sequence of DNA isolated *C. noctigama* UTEX114 (University of Texas culture collection) (K. Wilson, University of Saskatchewan unpublished results). Species of the genus *Chlamydomonas* are frequently used as model organisms, and the physiological and metabolic characters of some species of the genus are well-documented (Harris 1989; Harris 2001), including heavy metal tolerance and uptake (Bates *et al.* 1982; Hannikenne 2003; Mendez-Alvarez *et al.* 1999; Kobayashi *et al.* 2005).

Arsenic, nickel and uranium were chosen for this study because they are the main metallic contaminants of concern to Saskatchewan's uranium mining industry. Water used in this study was taken from the DJX pit in late August, 2004. From June to August of 2004, concentrations of arsenic, nickel and uranium in the DJX pit ranged from 0.0025-0.005 mg/L, 1.14-0.97 mg/L, and 2.914-2.233 mg/L, respectively (this study Appendix B, Table B-1; Dessouki 2005). Canadian Environmental Quality Guidelines for arsenic and nickel in freshwater specify that the surface water concentrations of these metals be less than 5 and 25-150 μg/L, respectively (CCME 2002). There is no set limit for uranium, and the uranium mining industry seeks to self-regulate in this regard and limits uranium concentrations to less than 100 μg/L (Cogema Resources Inc. 2000).

Four research objectives were addressed in this study. The first two were derived from questions that arose from research in the DJX pit in which nutrient additions produced sedimentation of metals from surface water (Dessouki 2005), but which lacked definitive proof that sedimentation was biologically mediated. Because results from the DJX field study varied dramatically between trials, the effect of phosphorus additions was also unclear. The first two objectives were:

1. To measure inorganic (physico-chemical) particulate matter and biotic sedimentation of arsenic, nickel and uranium to determine which is most important. (Chapter 2)
2. To determine if arsenic, nickel and uranium precipitation increases as phosphorus concentration increases, and if this correlates with algal cell density. (Chapter 2)

The third objective arose from the need for the direct demonstration of metal adsorption and metal uptake by cells:

3. To determine the type of mechanism that *C. noctigama* uses to sediment arsenic, nickel and uranium. That is, whether by sorption to cell walls, by uptake or by exudate. (Chapter 3)

The fourth objective arose from the convergence of classic limnological field procedures and emergent technologies such as those facilitated by synchrotron radiation. If Lugol's iodine were effective as a trace metal preservative for spectromicroscopy, there would be numerous historical samples that could be analyzed in this way. The fourth objective was:

4. To determine if algal samples stored in Lugol's iodine preservative can be used to assess trace metals by spectromicroscopy. (Chapter 4)

CHAPTER 2. STUDY OF UPTAKE AND SEDIMENTATION OF ARSENIC, NICKEL AND URANIUM BY *CHLAMYDOMONAS NOCTIGAMA*

2.1 Introduction

Uranium mining and milling operations produce a variety of metallic and other contaminants that are of concern to mine operators and regulators, as described in Chapter 1. After the extraction of ore from near-surface deposits the open pits fill with water. When these pits are decommissioned the mine operators must demonstrate that the water meets or exceeds national surface water quality guidelines (Lee *et al.* 1993). In most cases the contaminated water is pumped from the pit, treated chemically, and released into a nearby watershed, and the empty pit is refilled with uncontaminated water from a nearby lake. This method of decommissioning is costly, and does cause effects in the watershed where the treated water is released, such as increased salinity (Hynes 1990). However, there is evidence that over short periods of time (around 20 years) concentrations of toxic metals have naturally decreased to acceptable levels in the surface waters of flooded pits at two uranium mines, D-Pit at Cluff Lake mine and Gunnar Pit at Uranium City, without chemical treatment. It has been suggested that microbial populations, especially eukaryotic algae, may effect this decrease by sorbing or taking up metals in solution (Cogema Resources Inc. 2002; Dessouki *et al.* 2005). Then, when the algae subsequently senesce, die and sediment out of the water column, the metals are removed from surface waters.

The aim of the research summarized in this chapter was to confirm or refute that phytoplankton play a role in metal sedimentation from surface water, and whether this activity, if any, is enhanced by the addition of phosphate, as has been suggested by a previous field study (Dessouki *et al.* 2005). If phytoplankton do sediment metals from surface water, they could be used for remediation of water contaminated by metals.

I used a small-scale laboratory experiment to compare the removal and

sedimentation of arsenic, nickel and uranium[1] from contaminated water by a single algal species, *C. noctigama*, in relation to abiotic metal sedimentation in the absence of biota under the same conditions.

2.2 Methods

2.2.1 Experimental design and setup

The experiment was designed to permit comparison between treatments with and without cells along a phosphorus concentration gradient. The experimental design is shown in Figure 2.1. Two experimental trials were performed using this design. The second incorporated methodological and procedural refinements to improve the quality of results. In particular, there were more replicates in the second experiment to reduce sample variance, and there was a greater range in phosphorus concentrations in an attempt to produce a wider range of algal biomass along a phosphorus gradient. Procedural changes are summarized in Table 2.1 and described in detail in the text.

To minimize contamination of samples by biotic, metallic or other contaminants the following procedures were used (Acreman 2004). Culture inoculation was performed in a laminar flow hood and bottle mouths were flame sterilized. De-ionized distilled water was used for cleaning. Glassware and other tools were cleaned by washing in water with phosphorus-free detergent, rinsing with water, rinsing with methanol, re-rinsing with water, soaking for a minimum of 24 hours in an aqueous 5% hydrochloric acid solution, rinsing again in water, and air dried. Foam stoppers were cleaned by the same process. These were placed in the mouths of the flasks and the flasks were then capped with a square of aluminum foil before autoclaving. All glassware and tools were sterilized by autoclave.

Water for the experimental treatments was collected from the DJX pit during August and September, 2004. Water was collected in clean 20 L plastic containers and processed by filtration through 0.1 μm pore size cellulose acetate filters (from AMD Supply) to remove bacteria, algae and large colloidal matter from the water. Filtered

[1] Contaminants of specific interest to the uranium mining companies in Saskatchewan, AREVA and Cameco, are arsenic, nickel and uranium.

Phosphate additions:

Expt. 1:	0 ug/L	25 ug/L	50 ug/L
Expt. 2:	0 ug/L	35 ug/L	70 ug/L

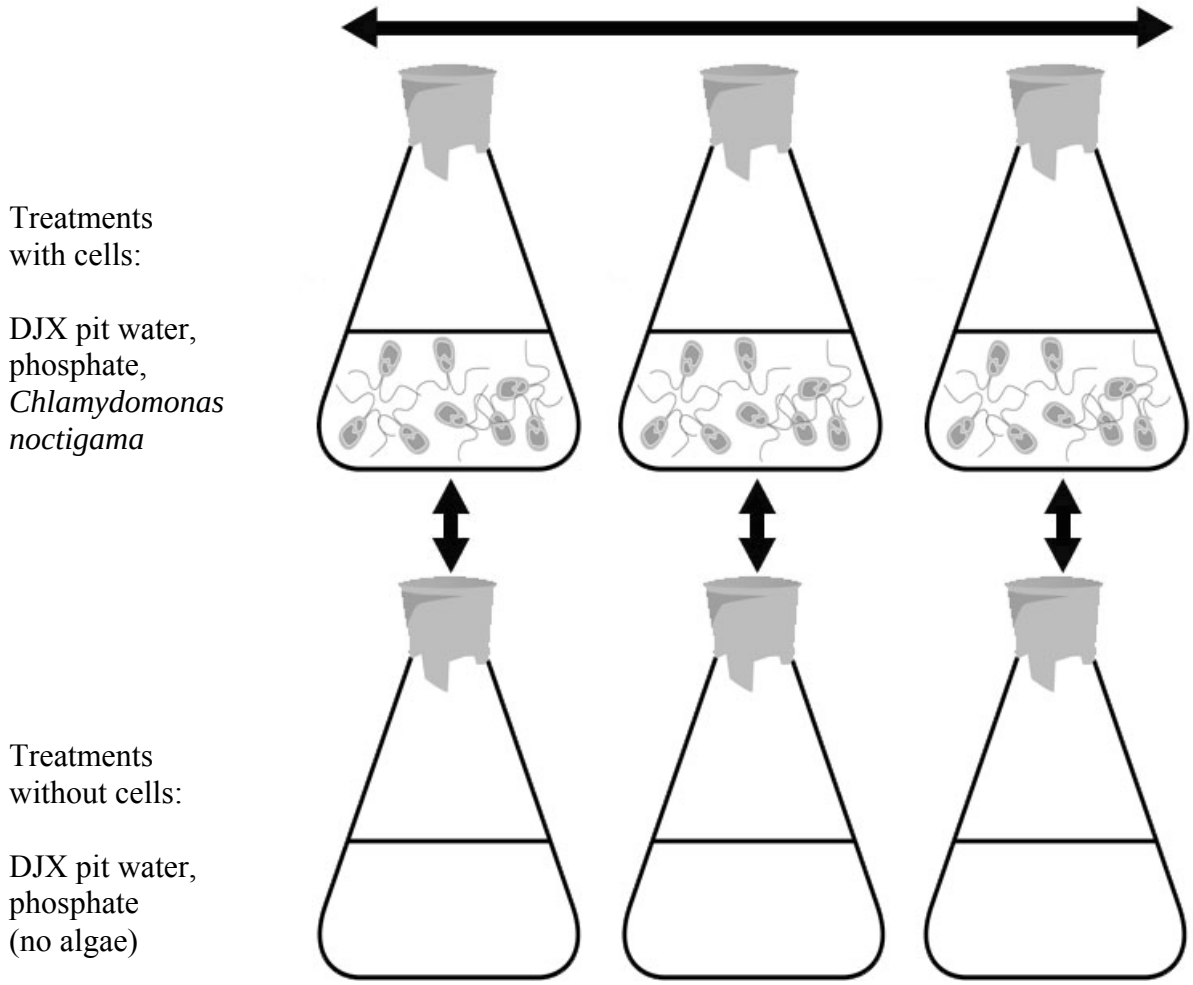


Figure 2.1. Experimental design for experiment 1 and experiment 2. Arrows represent possible comparisons between treatments with different phosphorus levels, and between treatments with and without cells.

Table 2.1. Comparison of experiments 1 and 2.

	Experiment 1	Experiment 2
Number of treatments	6 †	6 †
Number of replicates per treatment	4 ‡	7 *
Phosphorus additions	0 µg/L 25 µg/L 50 µg/L	0 µg/L 35 µg/L 70 µg/L
Temperature	25 °C	10 °C
Light	50 µmol/m^2/s	50 µmol/m^2/s
Duration	16 days	Cell cultures: 24 days No-cell "cultures": 25 days

† (1 treatment with cells + 1 treatment with no cells) x (3 P levels) = 6 treatments
‡ 3 replicates to ICP-MS and 1 for SEM and spectromicroscopy
* 5 replicates to ICP-MS and 2 for SEM and spectromicroscopy

water was stored in a clean 100 L barrel and was filtered again through 0.1 μm pore size cellulose acetate filters to ensure sterility before use. Following this second filtration, and prior to each experiment, water was stored in sterile, clean 4 L sidearm flasks capped with wax film to prevent contamination.

Phosphorus was added as monobasic potassium phosphate (KH_2PO_4) (Dessouki *et al.* 2005; Dessouki 2005). Phosphorus amendment concentrations include only the phosphorus component of the KH_2PO_4. The KH_2PO_4 was dried in a dessicator at 60 °C for 48 hours before it was weighed and added to a measured volume of DJX water to obtain a solution of known concentration. The specific concentrations used in the experiments were obtained by serial dilution of this solution. These solutions were sterilized by filtration through 0.1 μm filters. A 10 mL volume of KH_2PO_4 solution from the appropriate serial dilution was added to each of the experimental flasks that required phosphorus amendment. In the case of the treatments with no phosphorus amendment, a 10 mL volume of sterile DJX water was added. In both experimental trials, the zero phosphorus amendment treatments contained only the phosphorus already present in the DJX water. The three phosphorus concentrations in the growth media were designed to approximate oligotrophic (the zero phosphorus treatment), mesotrophic and eutrophic waters. pH was circumneutral (pH 7.0 to 7.5) in all treatments.

C. noctigama isolated from DJX pit water was used as the experimental organism in axenic culture for both experimental trials. Inoculant cultures were grown in DJX water at 5 °C to a high cell density, indicated by the rich green colour of the culture. Several large centrifuge tubes were each filled with 100 mL of culture, centrifuged at 8000 *g* for 10 minutes, and then 90 mL of the supernatant fluid from each tube was removed by pipette and the pelleted cells were re-suspended by gentle agitation. The contents of all centrifuge tubes were combined to provide the inoculant used for the cell treatments. During the experimental set-up, the inoculant was stored on ice to prevent thermal equilibration with the room temperature air in the laminar flow hood and resultant temperature shock to the cells. A sample of inoculant was placed in an amber glass bottle and preserved with Lugol's iodine for cell counts.

Cultures were grown in 250 mL borosilicate glass wide-mouth Erlenmeyer flasks. Following cleaning of flasks, as described above, flasks were dried at 60 °C for

24 hours, numbered and weighed. Then 150 mL of test solution was transferred to each flask, the inoculant was added and the flask mass was measured again. The same procedure was followed for the treatments without cells, except that the inoculant was replaced with filter-sterilized DJX water. At this time, culture flasks were set to grow under white light at an irradiance of 50 μmol/s at a schedule of 14 hours light to 10 hours dark. For the first experiment, flasks were arranged in random order on shelves at a uniform distance from a bank of fluorescent lights. Irradiance was measured at the position of each flask to ensure that each flask received equal illumination. For the second experiment, the six treatments were randomized within each of seven blocks on the shaker. Irradiance was measured directly above each flask to ensure that they received equal illumination from the overhead fluorescent lights.

For the first experiment, cultures were grown at ambient temperature and were manually agitated once daily for the duration of the trial. For the second experiment, cultures were continually agitated at 90 rpm on a shaker in a temperature controlled chamber at 10 °C. When, after a month, growth was not evident, cultures were re-inoculated with 1 mL of inoculant and shaking was discontinued. The average concentration of cells in the inoculant was 2450 (± 868) cells per mL (n = 6). After the second inoculation, growth was visibly evident within 10 days and the cultures were permitted to grow for 24 days.

2.2.2 Sampling methodology

At the end of each experimental trial, samples were processed as synchronously as possible. For experiment 1, all samples were processed in one day. For experiment 2, samples from cell cultures were taken one day and samples from "cultures" without cells were taken the following day. All sampling equipment and materials were trace metal clean and sterile. Work was performed in a laminar flow hood.

Because flasks were not sealed to air, water volume in flasks was expected to decrease by evaporation over the duration of the experiment. Determination of evaporative loss during the experiments was performed differently in the two experiments. In experiment 1, the volume of water in each flask was measured using a

graduated cylinder after the particulate matter was removed by filtration. In experiment 2, water loss was measured by the reduction in mass from the beginning to the end of the experiment.

Phosphorus determination was performed differently in experiment 1 than in experiment 2. In experiment 1 flasks were gently agitated till the contents were uniformly suspended, then 50 mL was removed by pipetting and placed in borosilicate glass tubes with polycarbonate caps for the determination of total phosphorus (i.e. dissolved and particulate). In experiment 2, 50 mL of filtered solution was placed in the tubes for the determination of dissolved phosphorus. Phosphorus was measured following the procedure described in Wetzel and Likens (2000). Samples from the first experiment were contaminated by incorrect processing and so were not useful, but the samples in the second experiment were processed correctly and phosphorus concentrations were obtained. Phosphorus concentrations were corrected for evaporative loss.

Cells and other particulate matter were separated from solution by filtration through 0.1 μm filters. Flask contents were gently agitated till the contents were uniformly suspended before filtration. Filtrate was collected in side-arm flasks, and processed into bottles for metal analysis by ICP-MS. In experiment 1, three of four replicates and in experiment 2, five of seven replicates of each treatment were prepared for ICP-MS. Filtered cells and particulate matter were packaged individually in pre-weighed filter holders, then, in experiment 1, dried at 60 °C for 48 hours. In experiment 2, filters were placed in a dessicator at room temperature for one week before mass measurement.

The mass of metals in water (i.e. dissolved metals) and on filters (i.e. particulate metals) were measured by ICP-MS at the Saskatchewan Research Council's analytical lab. Three replicates in experiment 1 and five replicates in experiment 2 were submitted for each of the six treatments. Arsenic and uranium was reported in units of μg/L and μg/filter and nickel was reported in units of mg/L and mg/filter to two significant figures. Spiked samples and treatment blanks were prepared for both experiment 1 and experiment 2. Metal concentrations in spiked samples and treatment blanks were in

accordance with expected concentrations and indicated that analysis was accurate and that no contamination occurred during sampling.

The remaining filters (one in experiment 1 and two in experiment 2) were reserved for SEM, EDX and, in experiment 2, for X-PEEM analysis. In experiment 2, these filters were cut in half. Half of the filter was prepared for SEM and EDX analysis, and the other half prepared for X-PEEM. Sample preparation for SEM was very simple: after drying as described above, a small square of filter was mounted on an SEM stub and gold coated to a thickness of approximately 200 Å. (Preparation of samples for EDX and for X-PEEM is described in Chapter 3.)

SEM was done using a JEOL JSM-840A scanning microscope in the University of Saskatchewan Department of Geology Electron Microscopy lab. Filtered matter was imaged at a uniform magnification of 200x along a transect across each filter, beginning at one corner and moving diagonally to the opposite edge. The census survey of matter was done by superimposing a 10 x 10 grid over each micrograph and estimating the area covered by cells, crystalline particulate matter, and amorphous matter in the area covered by the grid (equivalent to 250 μm^2). The number of squares containing matter that covered greater than or equal to 50 % of a square were counted for each matter type. Thirty replicate counts were done for each filter in this manner. There was one replicate from each treatment for both experiments.

Statistical analyses were done with the aid of GraphPad Prism© (GraphPad Software Inc. 2005). Two-way ANOVA and linear regression analysis was used to relate metal concentrations to treatments with and without cells and to treatments at different phosphorus levels (Figure 2.1). Non-parametric data from the survey of matter was analyzed by using a Kruskal-Wallace test to compare median values of percent crystalline particulate matter between phosphorus levels for treatments with and without cells. Treatments with and without cells were tested separately. The t-test with Welch's correction was applied to compare treatments with and without cells by each phosphorus level.

2.3 Results

2.3.1 Biotic and abiotic sedimentation

The particulate mass, comprising the mass of cells and of other non-dissolved matter, collected on filters from cultures with cells was greater than the particulate mass from treatments with no cells (Figure 2.2). Two-way ANOVA showed that this difference was highly significant in both experiments (Expt. 1: $F_{(1,12)} = 27.08$, $P = 0.0002$; Expt. 2: $F_{(1,24)} = 66.48$, $P < 0.0001$). There was more particulate matter in experiment 1 than in experiment 2.

Particulate concentrations of arsenic, nickel and uranium were significantly higher in all cultures with cells compared to those without cells, indicating that the sedimentation of metals was enhanced by the presence of *C. noctigama* (Table 2.2, Figures 2.3 A and B, 2.4 A and B, 2.5 A and B). Note that in the case of arsenic, in all treatments without cells the concentrations were below the ICP-MS detection limit of 0.05 μg/L. In treatments with cells, three of nine observations in the first experiment and six of 15 observations in the second experiment were below the detection limit. All data below the detection limit were assigned a value of 0.025 μg/L (the median value between 0 and 0.05 μg/L) for statistical analysis.

Dissolved metals are the fraction of metals that are not sorbed to solid particulate matter [2] and so are not removed by filtration. Dissolved metal concentrations were significantly lower in treatments with cells than in treatments without cells in both experiments, signifying that metal removal from solution was enhanced by the presence of *C. noctigama* (Table 2.2, Figures 2.3 C and D, 2.4 C and D, 2.5 C and D). The only exception to this trend was uranium in the first experiment[3] (Figure 2.5 C) where the cell

[2] Dissolved metals are operationally defined (in this study) as metals that are not removed by filtration through filters with pore size 0.1 μm.

[3] Dissolved uranium concentrations in the first experiment are about half those of the second, and of other measurements of uranium in DJX water (Appendix B, Table B-1; Dessouki *et al.* 2005) I suspect that the samples were diluted prior to measurement because analysts at the lab where analysis was performed were concerned that uranium concentrations were too high.

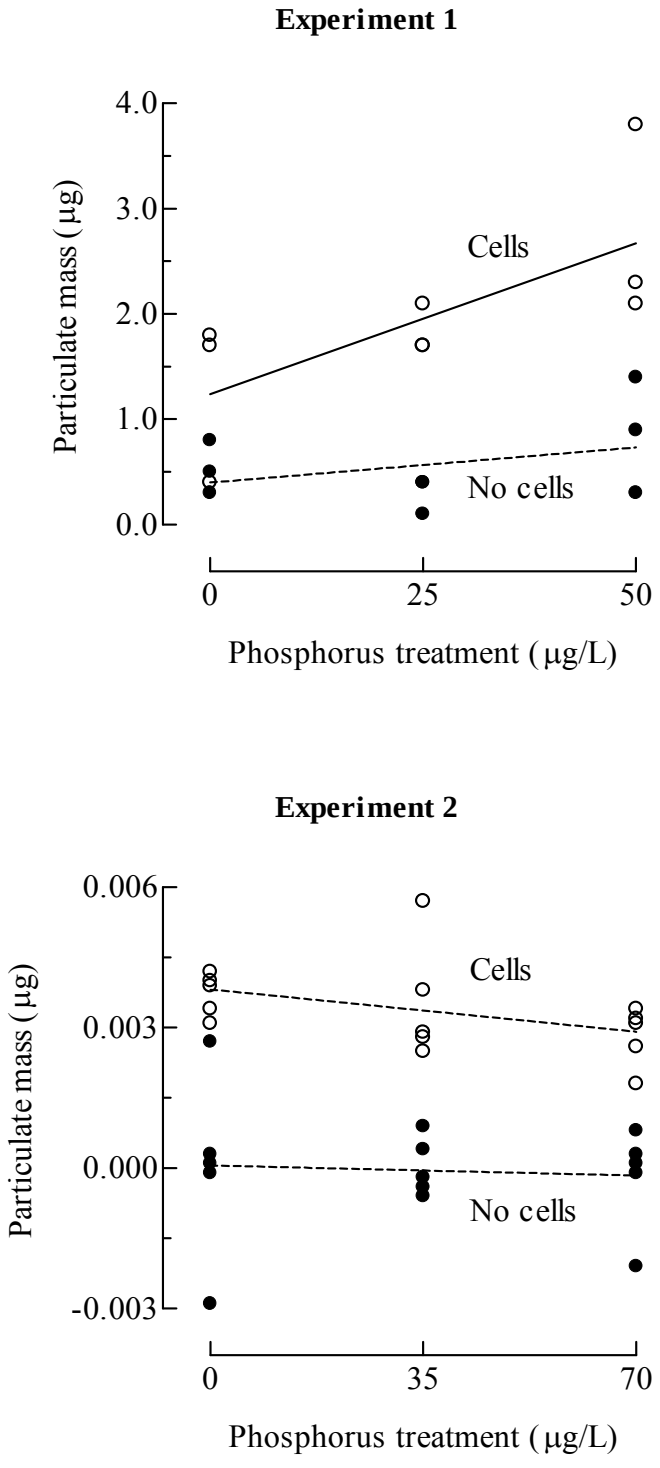


Figure 2.2. Particulate mass for treatments with cells (open circles) and without cells (closed circles) for experiments 1 and 2. Solid regression line indicates significant variation and dashed lines indicate non-significant variation.

Table 2.2. Summary of two-way analysis of variance results for dissolved and particulate metal concentrations.

Metal concentration in relation to:				
		Cell presence	Phosphorus level	Interaction
Particulate metal concentrations				
Arsenic	Expt. 1	+ cells higher (P = 0.015)	No effect (P = 0.18)	n.s.
	Expt. 2	+ cells higher (P = 0.0003)	No effect (P = 0.53)	n.s.
Nickel	Expt. 1	+ cells higher (P = 0.0002)	Positive correlation (P < 0.0001)	Significant (P = 0.001)
	Expt. 2	+ cells higher (P < 0.0001)	No effect (P = 0.27)	n.s.
Uranium	Expt.1	+ cells higher (P = 0.026)	Positive correlation (P = 0.0008)	n.s.
	Expt. 2	+ cells higher (P < 0.0001)	No effect (P = 0.49)	n.s.
Dissolved metal concentrations				
Arsenic	Expt. 1	+ cells lower (P = 0.0008)	No effect (P = 0.2)	n.s.
	Expt. 2	+ cells lower (P < 0.0001)	No effect (P = 0.12)	n.s.
Nickel	Expt. 1	+ cells lower (P < 0.0004)	Negative correlation (P = 0.002)	n.s.
	Expt. 2	+ cells lower (P < 0.0021)	No effect (P = 0.75)	n.s.
Uranium	Expt. 1	No effect (P = 0.28)	No effect (P = 0.09)	n.s.
	Expt. 2	+ cells lower (P < 0.0031)	No effect (P = 0.91)	n.s.

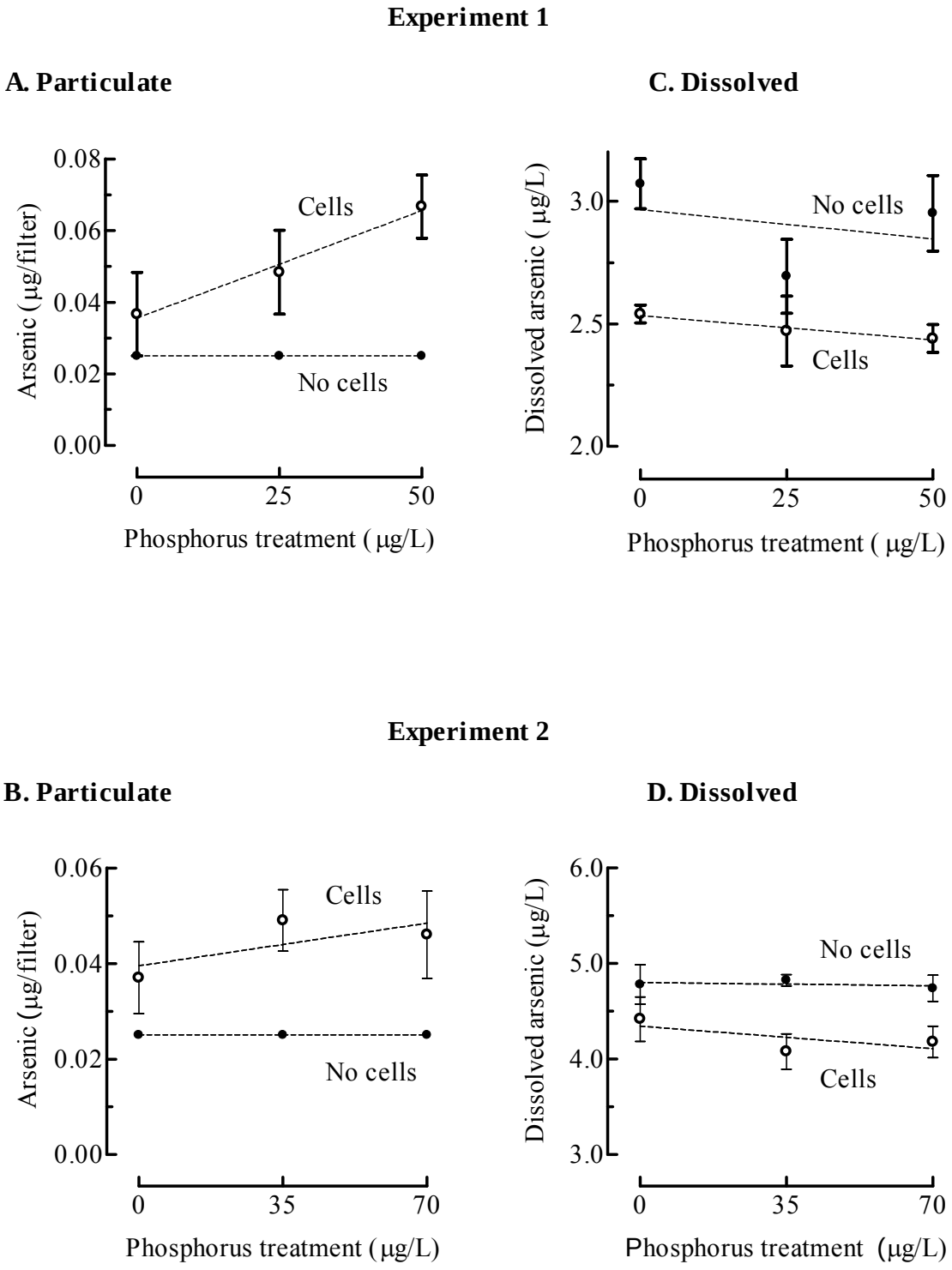


Figure 2.3. Mean particulate and dissolved arsenic for experiment 1 (A and C) and experiment 2 (B and D). Dashed regression lines indicate non-significant trends in relation to phosphorus.

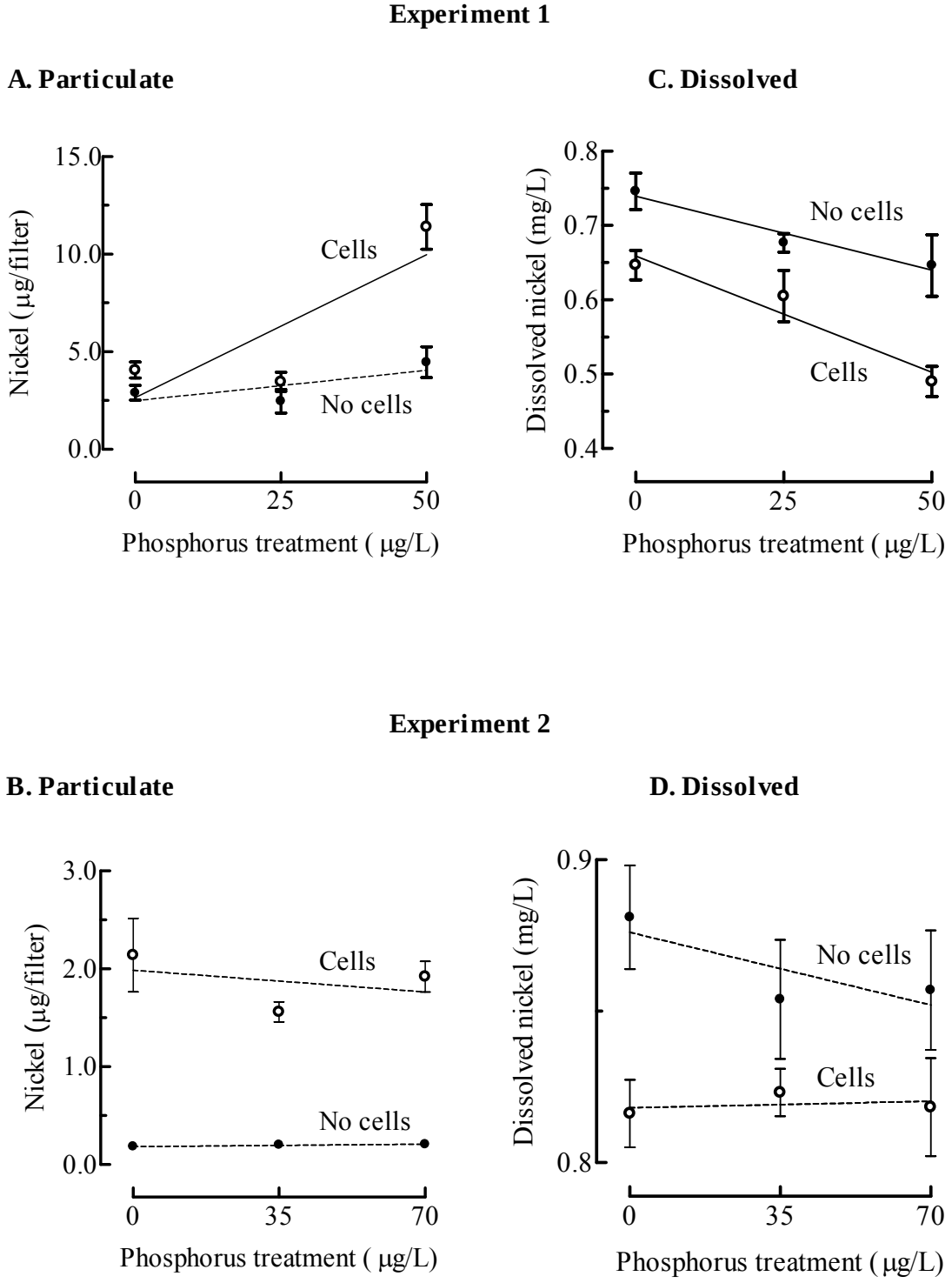


Figure 2.4. Mean particulate and dissolved nickel for experiment 1 (A and C) and experiment 2 (B and D). Solid regression lines indicate significant trends and dashed lines indicate non-significant trends in relation to phosphorus.

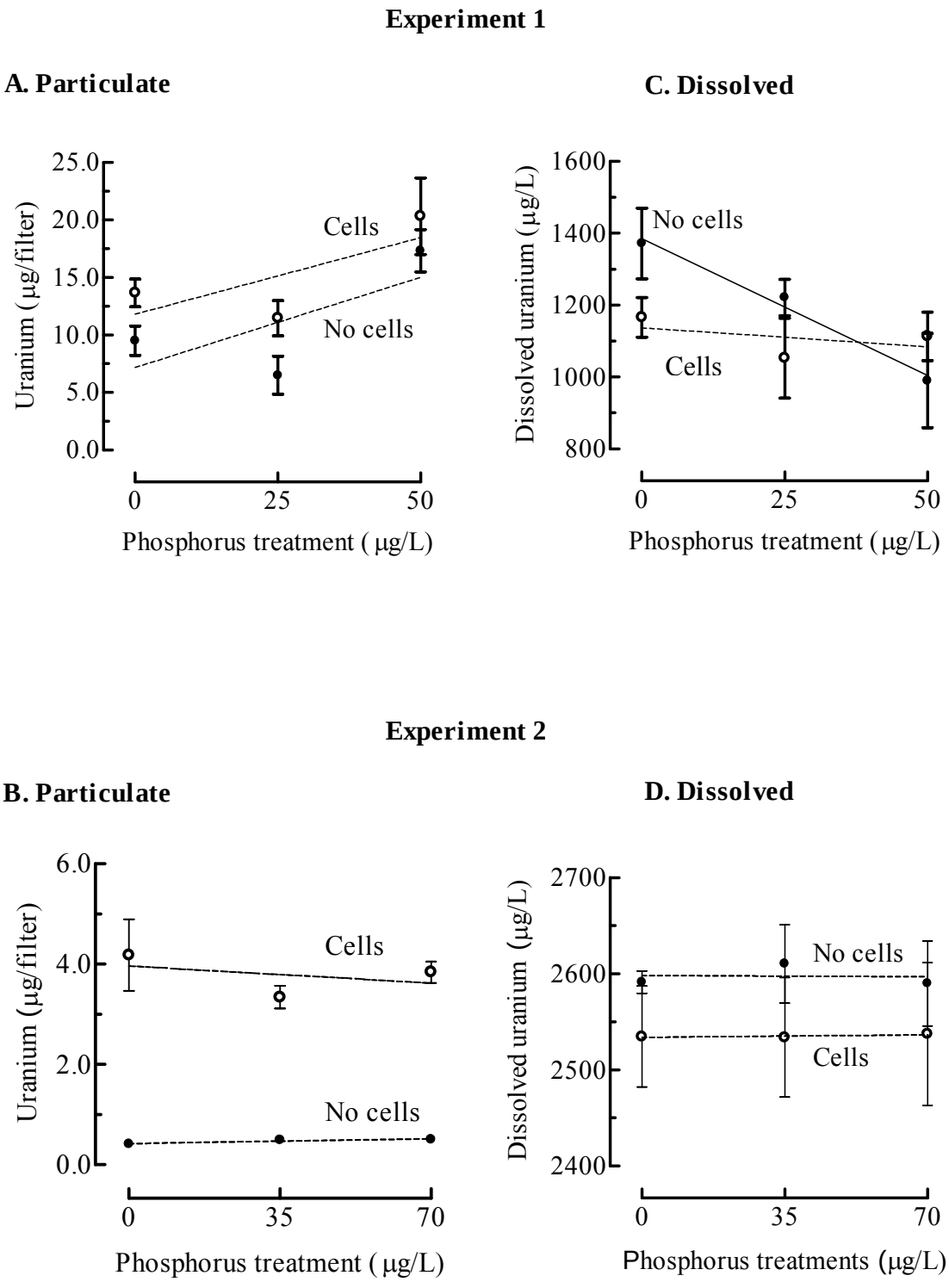


Figure 2.5. Mean particulate and dissolved uranium for experiment 1 (A and C) and experiment 2 (B and D). Solid regression line indicates significant trend and dashed lines indicate non-significant trends in relation to phosphorus.

and no cell treatments were not significantly different. However, this result is likely spurious, arising from the low level of replication in the first experiment (n = 3), which gave outliers undue weight in the statistical analysis.

In summary, the presence of *C. noctigama* significantly decreased the dissolved metal concentrations in the contaminated mine-water and consequently increased the amount of metals in particulate form (i.e. sedimented).

2.3.2 Sedimentation of arsenic, nickel and uranium in relation to phosphorus concentration

Particulate mass showed a significant increase with increasing phosphorus in the cell cultures in the first experiment ($F_{(1,7)} = 6.913$, $P = 0.0340$) but not in the second experiment ($F_{(1,13)} = 2.774$, $P = 0.1197$) (Figure 2.2). The significant regression in the first experiment was greatly influenced by two of the nine data points; in particular an unusually low value in the zero-phosphorus treatment and a single unusually high value in the highest phosphorus treatment. The remaining seven data points were all very similar so it is suspected that the relationship is spurious.

Particulate arsenic concentration was not influenced by phosphorus concentration in either experiment 1 or 2 (Table 2.2; Figure 2.3 A and C). Both particulate nickel and particulate uranium concentrations increased with increasing phosphorus concentration in the first experiment (Figures 2.4 A and 2.5 A), but not in experiment 2 (Figures 2.4 C and 2.5 C). It is suspected that these relationships are spurious for the reasons described above, and they were not confirmed in experiment 2 where there was more replication.

Dissolved arsenic concentrations (Table 2.2; Figure 2.3 B and D) and dissolved uranium concentrations (Figure 2.5 B and D) were unaffected by phosphorus levels or uranium concentration in either experiment 1 or 2. In experiment 1, dissolved nickel concentrations decreased with increased phosphorus (Figure 2.4 B), but this relationship is likely spurious for the reasons described above, and this result was not repeated in experiment 2 (Figure 2.4 D).

Table 2.3. Mean dissolved phosphorus concentrations for experiment 2 (s.d. in parentheses; n = 7).

Phosphorus treatment level	Cells	No cells
0 μg/L	2.52 (0.83) [1]	1.84 (0.26)
35 μg/L	2.48 (0.41)	1.64 (0.36) [1]
70 μg/L	2.35 (0.57)	1.17 (0.30)

1: (n = 6)

Phosphorus concentrations were high in all sample treatments (Table 2.3), including the treatment to which no potassium phosphate was added to the DJX water, indicating that phosphorus was not a growth-limiting nutrient in these experiments. Two-way ANOVA showed that dissolved phosphorus concentrations were significantly higher in treatments with cells than in treatments with no cells ($F_{(1,34)} = 34.04$, $P < 0.0001$), likely because the cell inoculum contained a large amount of phosphorus, but were not significantly different between phosphorus treatments ($F_{(2,34)} = 2.69$, $P = 0.0820$).

2.3.3 Scanning electron microscope survey of matter

Three distinct types of particulate matter on filters were observed on filters by SEM. Cells (Figure 1.2) were present only in treatments inoculated with *C. noctigama*. Crystalline particulate matter was present in all treatments, as was a non-quantifiable layer of amorphous particulate matter (Figure 2.6). Mean percent cellular and crystalline particulate matter at each phosphorus level for both experiments 1 and 2 are shown in Table 2.4. Experiment 1 results are far from clear, with t-tests indicating significantly more crystalline particulate matter in the presence of cells occurring only in the mid-range phosphorus treatment (Table 2.4, Table 2.5). While Kruskal-Wallace tests indicate variance by phosphorus level amongst the median values for crystalline particulate formed in the presence of cells, it is a non-linear variance (Table 2.6). In experiment 2, however, t-tests indicate that there was significantly more crystalline particulate matter in treatments with cells than in treatments without cells at all phosphorus levels (Table 2.4, Table 2.5), and Kruskal-Wallace tests showed clear variation among the medians for treatments with cells by phosphorus level for treatments with cells, but not for treatments without cells, not a surprising result since the median value for percent crystalline particulate matter at all phosphorus levels was zero (Table 2.6).

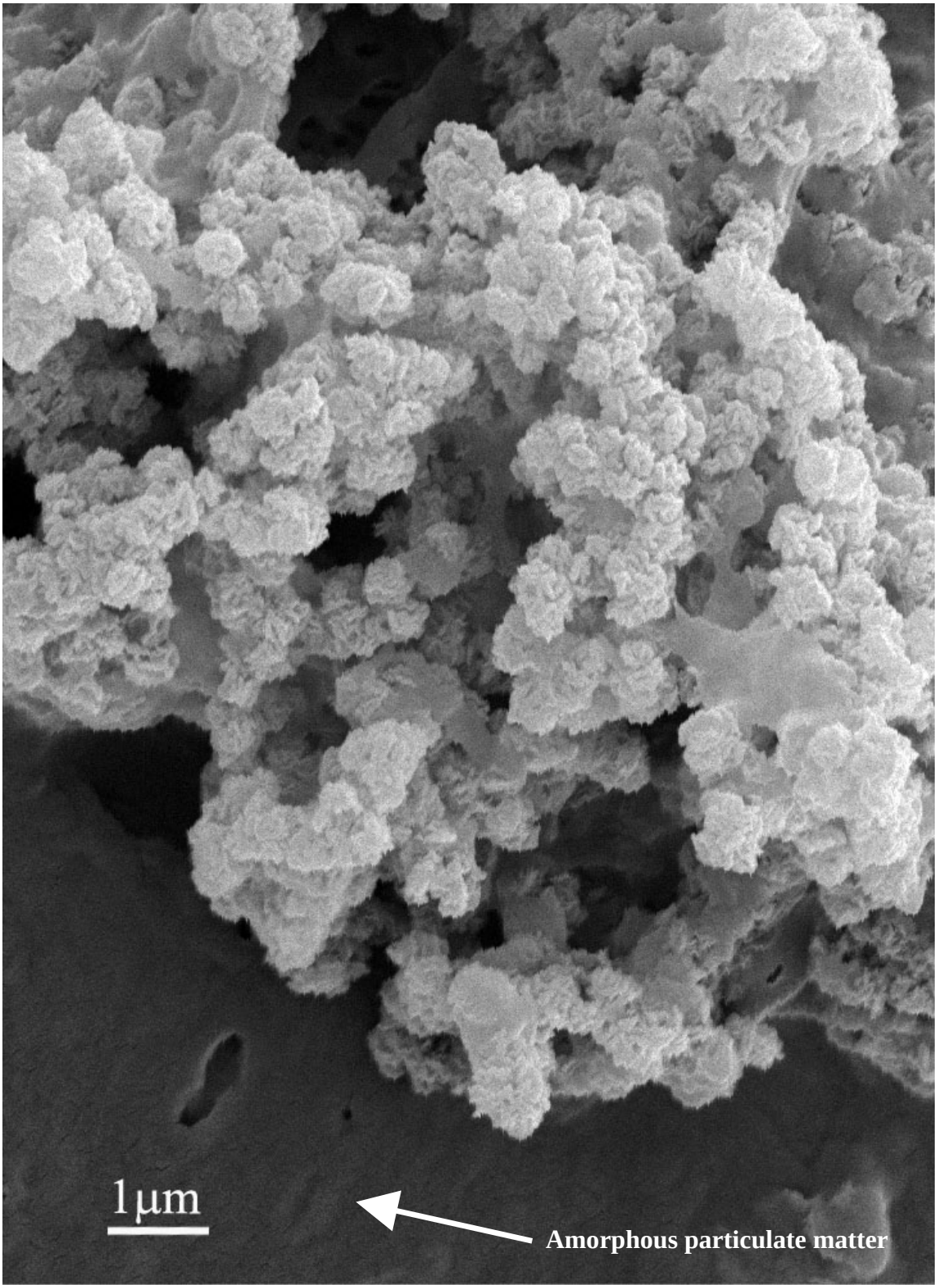


Figure 2.6. Scanning electron micrograph of crystalline and amorphous particulate matter.

Table 2.4. Mean percent cellular matter, crystalline particulate matter formed in the presence of cells and crystalline particulate matter formed in the absence of cells at each phosphorus treatment level for experiments 1 and 2 (sd in parentheses; 30 replicate counts in one sample; n = 1).

	Phosphorus treatment level	Percent cellular matter	Percent crystalline particulate matter in presence of cells	Percent crystalline particulate matter in absence of cells
Expt. 1	0 μg/L	5.4 (7.93)	7.3 (5.50)	7.8 (4.33)
	25 μg/L	3.4 (2.62)	14.5 (6.95)	5.8 (3.85)
	50 μg/L	3.4 (2.06)	4.6 (3.82)	6.5 (3.44)
Expt. 2	0 μg/L	0.37 (0.850)	3.4 (4.90)	0.067 (0.254)
	35 μg/L	1.0 (1.70)	4.7 (2.60)	0.50 (1.41)
	70 μg/L	0.27 (0.583)	6.4 (2.53)	0.10 (0.305)

Table 2.5. Mean percent crystalline particulate matter for treatments with and without cells at each phosphorus treatment level for experiments 1 and 2 (30 replicate counts in one sample; n = 1), and results from t-tests (with Welch's correction, 2-tailed).

	Phosphorus treatment level	Percent crystalline particulate matter in presence of cells	Percent crystalline particulate matter in absence of cells	t-test result
Expt. 1	0 μg/L	7.3	7.8	N.S., P = 0.6971
	25 μg/L	14.5	5.8	Sig., P < 0.0001
	50 μg/L	4.6	6.5	Sig., P = 0.0406
Expt. 2	0 μg/L	3.4	0.067	Sig., P = 0.0008
	35 μg/L	4.7	0.50	Sig., P < 0.0001
	70 μg/L	6.4	0.10	Sig., P < 0.0001

Table 2.6. Median percent crystalline particulate matter for treatments with and without cells at each phosphorus treatment level for experiments 1 and 2 (30 replicate counts in one sample; n = 1), and results from Kruskal-Wallace tests.

Expt. 1			Expt. 2		
Phosphorus treatment level	Percent crystalline particulate matter in presence of cells	Percent crystalline particulate matter in absence of cells	Phosphorus treatment level	Percent crystalline particulate matter in presence of cells	Percent crystalline particulate matter in absence of cells
0 μg/L	6.0	8.0	0 μg/L	3.0	0.0
25 μg/L	12.5	4.5	35 μg/L	4.0	0.0
50 μg/L	3.5	6.5	70 μg/L	6.5	0.0
K-W test result	33.85 P< 0.0001 (Sig.)	4.136 P = 0.1264 (N.S)		28.95 P< 0.0001 (Sig.)	0.9848 P = 0.6111 (N.S)

2.4 Discussion

2.4.1 Uptake and sedimentation of metals by *Chlamydomonas noctigama*

The first aim of this research was to confirm or refute that phytoplankton play a role in metal sedimentation from surface water. My results clearly indicate that sedimentation of metals from DJX water was significantly enhanced by the presence of *C. noctigama*. Arsenic, nickel and uranium concentrations in particulate matter were significantly higher in treatments with cells than in treatments without cells, and dissolved concentrations were significantly lower in treatments with cells than in treatments without cells.

These results are consistent with the hypothesis that algae sorb or take up metals. Passive sorption and active uptake of many metals by algae and other cells is well-documented (Rangsayatorn *et al.* 2002; Harrison *et al.* 1986; Meylan *et al.* 2003; Mylon *et al.* 2003; Schenk *et al.* 1988; Chen and Folt 2005; Kauffman *et al.* 1986)(See Appendix A Table A-1.), and biosorption of arsenic has been observed in the fungus *Penicillium chrysogenum* (Loukidou *et al.* 2003), by the bacterium *Thiobacillus ferrooxidans* (LeBlanc *et al.* 2002) and by several chlorophyte algae including *C. reinhardtii* (Mahan *et al.* 1989). Nickel has been removed from water by *Escherichia coli* (Deng *et al.* 2003), the chlorophytes *Chlorella sorokiniana* (Akhtar *et al.* 2004), *Ankistrodesmus* sp. and *Selenastrum* sp. (Mann and Fyfe 1984), *Ulothrix* sp. (Lawrence *et al.* 1998), and *Chlamydomonas* sp. (Wang and Wood 1984). Uranium has been removed from water by the bacteria *Clostridium* sp. (Kauffman *et al.* 1986), *Pseudomonas aeruginosa* (Renninger *et al.* 2004), several sulfate-reducing bacteria (Spear *et al.* 2000) and the chlorophytes *Ankistrodesmus* sp. and *Selenastrum* sp. (Mann and Fyfe 1984; Mann and Fyfe 1985). Contradicting the abundant evidence supporting metal-algae associations, Suzuki *et al.* (2005) found uranium in association with bacterial cells but not with algal cells in shallow sediments of an open pit mine at an inactive uranium mine (the Midnite mine in Stevens County, Washington, U.S.A.), but that result may indicate that the algae do not persist in sediments in the long term.

There is a question of whether metal removal is by done by the cells directly or by complexation with cell products. In experiment 2, significantly more crystalline

particulate matter occurred in matter collected on filters from treatments with cells than from treatments without cells, possibly resulting from induction of production of crystalline particulate matter by *C. noctigama*. Metal-chelating algal cell products are well-documented (Ahner and Morel 1995; Lee *et al.* 1996; Dreschel and Jung 1998; Barker and Stuckey 1999; Leal *et al.* 1999; Davis *et al.* 2003) and likely serve as crystallization nuclei for mineralization of exogenous dissolved elements (Labrenz *et al.* 2000; Chan *et al.* 2004; Fortin 2004). Other polysaccharide exudates may serve the same function. The algal cell makes the crystallization template or nucleus and the constituents of the crystal are derived from the surrounding environment.

It is likely that the movement of metals from surface water to sediments observed by Dessouki *et al.* (2005) during mesocosm studies at the DJX pit was mediated by algae, and possibly bacteria, and not by abiotic precipitation. This is probably not a unique phenomenon, as sedimentation of heavy metals from surface waters has been documented in marine (Hunt 1983) and fresh water settings (Gächter 1979; Jackson *et al.* 1999; Sigg *et al.* 1987; Vignati and Dominik 2003). Suspended particles sediment when the buoyant force is exceeded by the force exerted upon them by gravity. Thus, surface water metal concentrations would decrease when dense co-aggregates of algae and suspended mineral and colloidal particulate matter (Avnimelech *et al.* 1982; Leppard *et al.* 2003; Vignati and Dominik 2003) sorb metals and fall from the surface water to the sediments.

2.4.2 The role of phosphorus in biotic and abiotic metal sedimentation

The second aim of this research was to determine the role of phosphorus in biotic and abiotic metal sedimentation. Based on these experiments, this role is still poorly characterized. In my experiments, phosphorus seemed to play no role in increasing biotic metal sedimentation. This is likely because the phosphorus concentration in the DJX pit water used was above the threshold that would limit algal growth, so that the addition of more phosphorus did not affect particulate mass in the experimental

cultures[4].

There are, however, several studies that connect metal accumulation by algae to the phosphorus concentration in media (Wang and Dei 2001a; Wang and Dei 2001b; Yu and Wang 2004; Dessouki *et al.* 2005). Because phosphorus has a low solubility in water and is highly reactive, it was suspected that phosphorus might play a role in inorganic metal sedimentation in experimental media by increasing precipitation of compounds such as uranyl phosphates and ningyoite ($CaU(PO_4)_2$) (Denecke *et al.* 2005; Langmuir 1978; Morel and Hering 1993). In this study, I analyzed a single sample from each phosphorus level from both experiments and showed a significant correlation between phosphorus level and the volume of inorganic matter generated in the form of crystalline particulate matter, with a linear increase in the median percent crystalline particulate matter by phosphorus level. Because this result is based on an analysis of unreplicated data, it may not indicate the true relationship between phosphorus level and formation of crystalline particulate matter. The main role of phosphorus in sedimentation of metals may simply be to encourage algal growth. If, however, phosphorus level and crystalline particulate formation do positively correlate, and if the crystalline precipitate has a metallic component there could be an associated increase in the sedimentation of metals. This aspect will be examined further in Chapter 3.

In summary, this study provides clear evidence that metal removal from mine-impacted water can be mediated by algae. Only one algal species, *C. noctigama*, was used in this study, but it is expected that experiments using other algal species, prokaryotic phytoplankton and bacteria would give similar results, based on the overall similarity of metal removal by phytoplankton observed in this study and by many other researchers in various independent studies. It is very likely that mining-impacted water could be remediated using phytoplankton, and that the observed improvements in the surface water qualities of the Gunnar Pit near Uranium City and the D-Pit at Cluff Lake uranium mine are largely the result of the action of phytoplankton. The improved water

[4] When dramatic decreases in surface water metal concentrations were observed during Dessouki's 2003 field trials, a large amount of phosphorus was added to the water of DJX pit (in the Fall of 2003) to increase the surface water phosphorus concentrations and induce sedimentation of metals (Dessouki 2005). In the summer and fall of 2004, when water for these experiments was obtained from DJX pit, phosphorus concentrations were still very high.

quality persists because of meromixis, due to the morphometric character of these pit lakes, i.e. small surface area to depth ratios and low fetch. However, our understanding of the role of phosphorus in increasing the rate of metal sedimentation remains incomplete. When phosphorus is limiting, an increase in phosphorus leads to an increase in algal growth and an increased sedimentation of metals (Dessouki *et al.* 2005). When phosphorus is no longer a limiting factor for algal growth, however, as was the case in my experiments, there may be little advantage to increasing phosphorus concentrations still further. Indeed, I did not find convincing evidence for an increase in metal sedimentation as more phosphorus was added to the experimental cultures.

CHAPTER 3. SPECTROMICROSCOPIC INVESTIGATION INTO THE NATURE OF METAL UPTAKE AND PRECIPITATION BY *CHLAMYDOMONAS NOCTIGAMA*

3.1 Introduction

Metal removal and sedimentation increases in the presence of *Chlamydomonas noctigama*, likely due to some cell-mediated process (Chapter 2). There are several ways that *C. noctigama* might have mediated the observed metal sedimentation from solution. Extra-cellular binding occurs passively by the interaction of positively-charged metals with negatively-charged moieties on the cell surface. In addition to providing a surface for metal sorption (Schulz-Baldez and Lewin 1975; Bates *et al.* 1982; Mann and Fyfe 1985; Jackson *et al.* 1999; Fowle *et al.* 2000; Kelly *et al.* 2001; Rai and Gaur 2001; Rangsayatorn *et al.* 2002; Merroun *et al.* 2005), cell walls also serve as a barrier to metal ion uptake into cells (Macfie and Welbourn 2000), but wall-bound transport proteins permit active uptake of metals (Harrison *et al.*1986; Sunda and Huntsman 1998; Fortin and Campbell 2001; Rai and Gaur 2001; Mulrooney and Hausinger 2003; Hannikenne *et al.* 2005; Kobayashi *et al.* 2005; Kola and Wilkinson 2005; Rosakis and Köster 2005). Many algae and other microorganisms may also induce production of metal-chelating compounds in response to metal stress (Rijstenbil *et al.* 1998; Sunda and Huntsman 1998; Mejáre and Bülow 2001; Rai and Gaur 2001), and these chelating compounds may aid in the intracellular sequestration or metabolism of metals and metalloids in vacuoles or as crystals in the cell (Schenck *et al.* 1988; Howe and Merchant 1992; Hill *et al.* 1996; Dreschel and Jung 1998; Gonzalez and Jensen 1998; Mehta and Gaur 1999; Watt and Ludden 1999; Siripornadulsil *et al.* 2002; Hellweger *et al.* 2003; Leppard *et al.* 2003), which may mediate the export of metals from the cell after uptake (Ahner and Morel 1995; Lee *et al.* 1996; Langley and Beveridge 1999; Labrenz *et al.* 2000; Rai and Gaur 2001; Chan *et al.* 2004; Renninger *et al.* 2004), or which may directly complex the metals in dense precipitates outside of the cells (Leal *et al.* 1999; Liu *et al.* 2001; Liu *et*

al. 2002; Leppard *et al.* 2003). It is possible that more than one mechanism was used in the removal and sedimentation of arsenic, nickel and uranium from solution observed and documented in Chapter 2, and that each of these metals is subject to differing mechanisms of removal.

Direct observation of metal localization in, on and near cells would provide increased understanding of how this uptake and sedimentation occurs. However, because cells of *C. noctigama* are small (2-10 μm), and because the concentrations of the metals of interest are anticipated to be very low in the cells, it is impossible to discern any interactions between the metals and the cells using conventional quantitative means of detection such as ICP-MS. Spectromicroscopic techniques, such as energy dispersive X-ray spectroscopy (EDX) and X-ray photoelectron emission microscopy (X-PEEM), have been used to view the localization of trace elements in very small samples. EDX provides an assessment of the presence of all of the elements in samples of intermediate size (of area greater than 1 μm^2), and relative concentrations of elements in samples can be estimated by comparison of peak strengths in spectra, although definitive quantification of metal concentrations is difficult due to X-ray absorption and fluorescence effects (Bauer *et al.* 1995). In combination with scanning electron microscopy (SEM) and transmission electron microscopy (TEM), EDX has been used to observe trace metals present at high concentrations, including arsenic, nickel and uranium, in diverse studies (Klich *et al.* 2001), including in biological samples (Bhatia *et al.* 2003; Merroun *et al.* 2005) and in samples of biota-associated mineral complexes (Schulz-Baldes and Lewin 1975; St-Cyr *et al.* 1993; Merroun *et al.* 2002; Renninger *et al.* 2004; Suzuki *et al.* 2005). Manganese encrustations have also been observed on the surfaces of *Chlamydomonas* zygospores with EDX.

X-PEEM has high spatial resolution (up to 6 nm (Frazer *et al.* 2004)) and tunability, which permits mapping of elements within very small regions of interest and the determination of detailed elemental speciation information from regions of interest (Hitchcock *et al.* 2002).

Both of these techniques were used to begin to explore the types of mechanisms that *C. noctigama* uses to sediment arsenic, nickel and uranium, that is, whether by

sorption to cell walls, by uptake or by exudate, or by some combination of these mechanisms.

3.2 Methods

3.2.1 EDX spectroscopy

Spectral data was obtained by EDX analysis of cells (Figure 1.2), and crystalline particulate matter and amorphous matter (Figure 2.6) in samples of filtered particulate matter from the metal uptake experiments described in Chapter 2. One replicate of each treatment from experiment 1 and two replicates of each treatment from experiment 2 were used. A small square excised from each filter was coated with 200 Å of carbon prior to EDX. EDX was performed on the SEM instrument Hitachi S-3000N and Oxford INCA Analyzer-ISIS/INCA (Oxford Instruments Inc. 2005) at the Saskatchewan Research Council (SRC) Geoanalytical laboratory. In EDX analysis, the electron beam is focused on a chosen area of the sample, and detectable elements in the irradiated region emit electrons at characteristic energies, permitting determination of abundances of elements in the sample. For this experiment, samples were scanned over the energy range from 0 to 20 keV, in increments of 0.01 eV. Spectra were obtained from cells, crystalline and amorphous particulate matter in each sample, over a 1 μm by 1 μm area of the sample. Five replicate scans were done for each matter type on each sample (where possible). From treatments with cells from all phosphorus levels, 44 cell spectra, 45 crystalline particulate matter spectra and 38 amorphous matter spectra were obtained. From treatments without cells, 18 crystalline particulate matter and 30 amorphous particulate matter spectra were obtained. Presence or absence of the elements aluminum, arsenic, calcium, chlorine, cobalt, copper, iron, magnesium, manganese, molybdenum, sodium, nickel, phosphorus, sulfur, uranium and zinc[5] in the spectra was determined by comparison to characteristic photon energies and relative intensities of K-, L- and M-shell lines over the range of 0 to 20 keV (See Appendix C) in Igor Pro 5.04B graphing software (WaveMetrics 2005). Peak strength was not considered in this analysis so no

[5] These elements were selected either because they are present in samples from the DJX pit at Cluff Lake mine, because they are common contributors to cell mass, or because they are likely contributors to biomineral complexes.

conclusions as to the relative concentrations of elements will be made based on this analysis. To distinguish the role of each matter type in the uptake and sedimentation of As, Ni, U and co-occurring elements, the G-test of independence (Sokal and Rohlf 1995) was used to compare frequencies of occurrence of each element between cells, crystalline and amorphous particulate matter in treatments with cells, and between crystalline and amorphous particulate matter in treatments without cells. Frequencies of occurrence of elements were also compared between crystalline particulate matter from treatments with and without cells, and between amorphous particulate matter from treatments with and without cells.

In addition to the analysis of frequencies of elemental occurrence, described above, the peak strengths of individual elements occurring in spectra from each matter type were compared to determine the dominant components of each matter type.

3.2.2 X-PEEM spectroscopy

Maps and spectra of elemental locations in a cell were obtained by X-PEEM, on the SPHINX (Spectromicroscope for photoelectron imaging of nanostructures with X-rays) instrument at the Synchrotron Radiation Center (SRC) in Stoughton, Wisconsin. The SPHINX spectromicroscope operates on the VLS-PGM (varied line space plane grating monochromator) beamline at SRC, which has an energy range of 75 to 2000 eV.

Live cells from the stock cell culture (cells grown in DJX water with no nutrients added) were used for X-PEEM microscopy. Cells and water were placed in a dialysis bag constructed of a folded 0.1 μm pore size cellulose acetate filter, and suspended in a beaker of de-ionized, distilled water for 12 hours. This was done to dissolve salts which obscured cells in early attempts. Subsequently, a droplet of the cells and dilute DJX water in the dialysis bag was pipetted onto a 1 cm^2 silicon slide and allowed to air dry. The sample was sputter-coated with 12 Å of platinum, placed in a sample holder, and sealed for transport to the SPHINX spectromicroscope. A *C. noctigama* cell was located in the sample, and images, maps and movies were taken from it. Uranium was detected by scanning over the range 120 to 92 eV at 0.4 and 0.1 eV steps. Additionally, carbon was detected by scanning over the energy range 315 to 275 eV at 0.5 and 0.1 eV

intervals, and phosphorus over the range 160 to 130 eV at intervals of 0.2 eV. Pixelated movies resulting from the varying emission energy intensities from the cell and surrounding area were compiled by the SPHINX operating software.

Spectra were extracted from the movies obtained using NIH *Image* Version 1.63 (Research Services Branch, National Institutes for Mental Health 2005), using macros written by the Gilbert Group at the University of Wisconsin.

3.3 Results

3.3.1 EDX spectroscopy results

It is important to note that I used two types of information from the EDX spectra. Elements in the sample show up in the spectra as a set of peaks at characteristic locations for those elements. The height of the peaks relative to other peaks indicate relative concentrations of elements. In EDX data analysis, relative peak height is an indicator of relative concentrations of elements, and in my analysis I considered the presence of a peak of any height an indicator of the presence of the element in the sample. They do not necessarily correspond, however. For example, where there was a high rate of occurrence of an element in a particular sample type (i.e. there were peaks characteristic of the element in many samples of that type), the peaks for that element might not be very high. Nonetheless, since each measure indicates a different thing, both are useful to understand the nature of a sample.

Comparison of selected representative spectra from a cell, from crystalline particulate and from amorphous particulate matter (Figures 3.1 and 3.2) shows that concentrations of the various elements are generally highest in crystalline particulate, followed by cells, with amorphous particulate showing the lowest concentrations. (Peak intensity or height estimates concentration. See Appendix C, Table C-1 for energies and intensities of emission lines for selected elements of interest and for elements that occur within the scanned range).

In the presence of cells, metal frequency ranked highest in crystalline precipitate matter for 10 of the 15 elements studied, and were significantly more frequent than in cells for seven elements (Table 3.1). Metal frequency ranked highest in cells for five of

the 15 elements, but was never significantly higher than in crystalline particulate. Frequency in amorphous particulate was higher than in cells for two elements, but was never higher than in crystalline precipitate (Table 3.1). In the absence of cells, metal frequency ranked higher in crystalline particulate matter than in amorphous particulate in 13 of the 15 elements studied, though the differences were only significant for three elements (aluminum, calcium and chlorine) (Table 3.2). Thus, metals tended to be more closely associated with crystalline particulate than with cells or amorphous particulate matter.

The potential role of cells in the precipitation of metals is more difficult to elucidate. Consider first the three metals of primary interest. Arsenic was more frequent in both cells and crystalline particulate compared to in amorphous particulate (Table 3.1), and so cells clearly play a role in removing arsenic from solution. Nickel and uranium were significantly most frequent in crystalline precipitate in the presence of cells (Table 3.1) but not in the absence of cells (Table 3.2), indicating that cells enhance the precipitation of these metals. However, there was no significant difference in the frequencies of occurrence of these elements when crystalline particulates were compared from treatments with and without cells (Table 3.3), which does not support the suggestion that cells enhance the precipitation of these three metals in crystalline form.

Six other metals (aluminum, calcium, iron, magnesium, manganese and sodium) were most frequent in crystalline particulate in the presence of cells (Table 3.1), and only aluminum and calcium were also most frequent when no cells were present (Table 3.2), suggesting that cells promote the precipitation of iron, magnesium, manganese and sodium in crystalline form. This is supported for iron, magnesium and manganese because they were significantly more frequent in crystalline particulate in cell cultures compared to in cultures without cells (Table 3.3).

Phosphorus occurred significantly more frequently in cells (Table 3.1), as might be expected, and was also significantly more frequent in both crystalline particulate (Table 3.3) and amorphous particulate (Table 3.4) in cell cultures compared to cultures without cells.

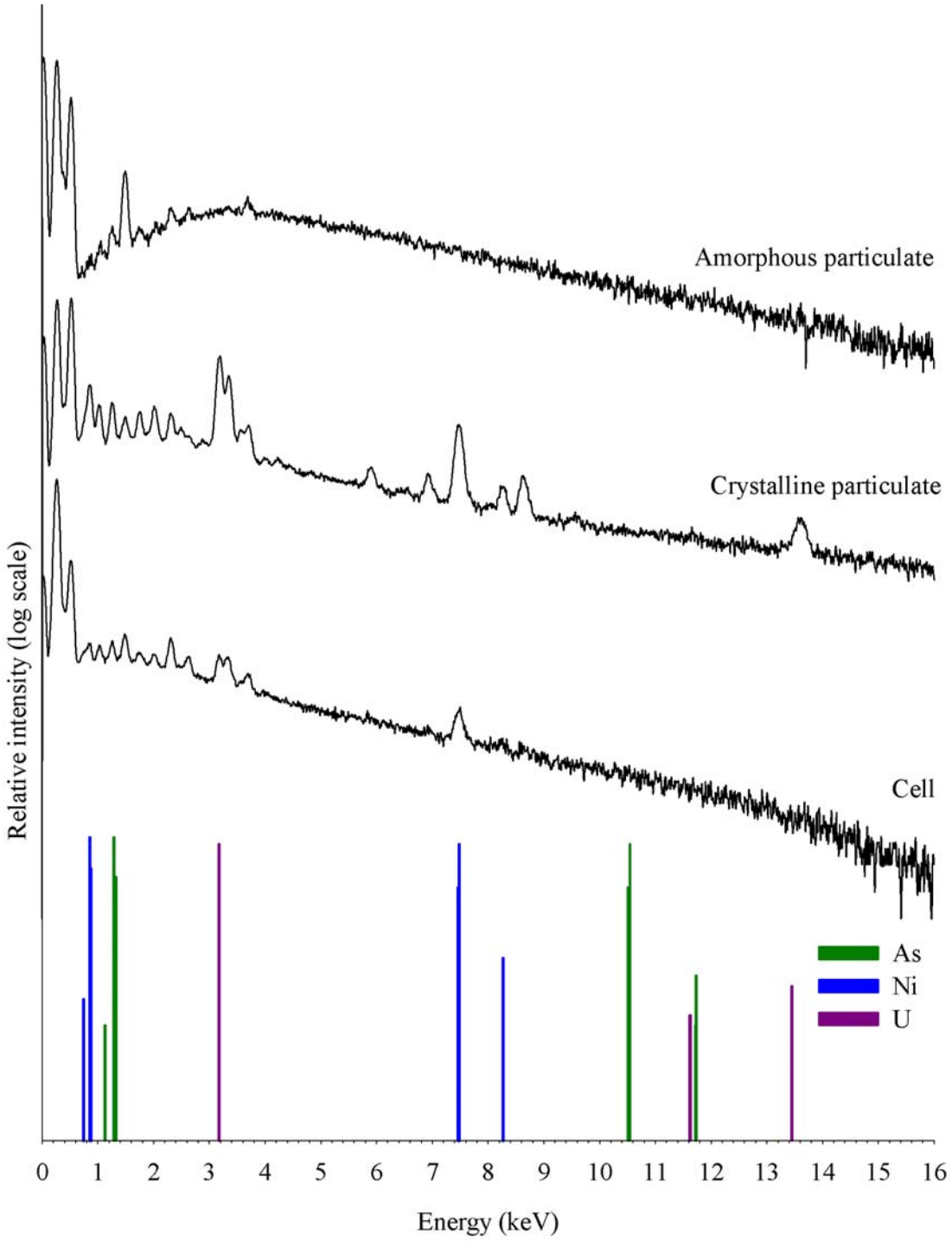


Figure 3.1 EDX spectra from a cell, crystalline particulate matter and amorphous particulate matter showing the relative intensities (log scale) of contributing elements over the range of energies from 0 to 16 keV, and characteristic relative intensities of emission lines for the elements arsenic, nickel and uranium in this range. Spectra are offset on the Y-axis to facilitate comparison.

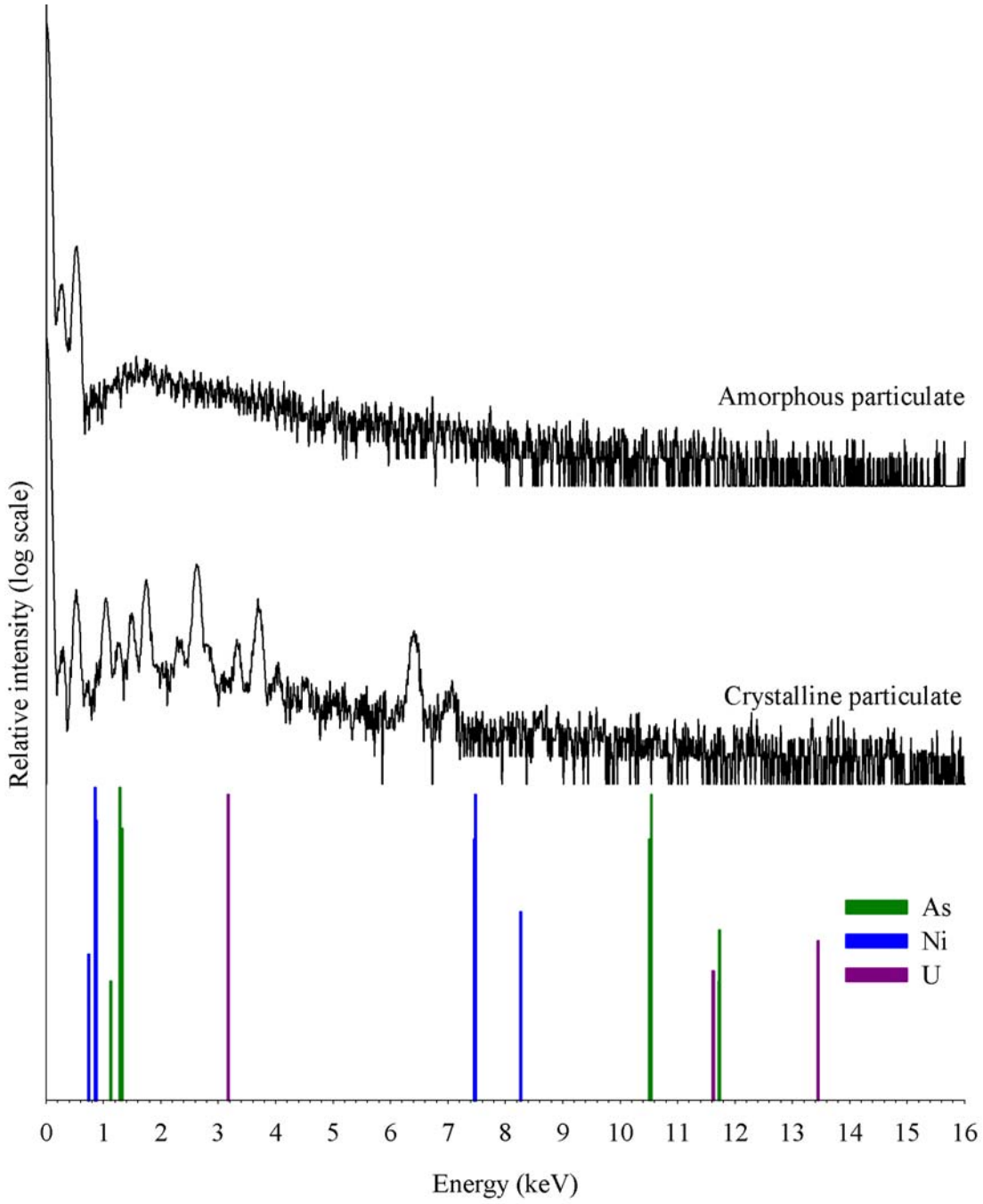


Figure 3.2 EDX spectra from crystalline particulate matter and amorphous particulate matter showing the relative intensities (log scale) of contributing elements over the range of energies from 0 to 16 keV, and characteristic relative intensities of emission lines for the elements arsenic, nickel and uranium in this range. Spectra are offset on the Y-axis to facilitate comparison.

Table 3.1 Frequencies of occurrence of selected elements in cells, crystalline particulate matter and amorphous particulate matter from treatments with cells. Asterisks indicate significant differences.

Element	Cells (a) (n = 44)	Crystalline particulate matter (b) (n = 45)	Amorphous particulate matter (c) (n = 38)	Significant differences (G-test values in parentheses)*	
Aluminum	0.70	0.87*	0.68	b > c	(4.065)
Arsenic	0.68*	0.60*	0.26	a > c	(14.783)
				b > c	(9.712)
Calcium	0.82	0.98*	0.87	b > a	(6.988)
				b > c	(3.897)
Chlorine	0.75	0.67	0.68		
Cobalt	0.57	0.58	0.37		
Copper	0.41	0.33	0.34		
Iron	0.48	0.87*	0.39	b > a	(16.106)
				b > c	(21.091)
Magnesium	0.93	0.98*	0.79	b > c	(8.271)
Manganese	0.23	0.96*	0.53*	b > a	(56.585)
				b > c	(22.726)
				c > a	(7.962)
Molybdenum	1.00	0.96	0.92		
Nickel	0.48	0.91*	0.68	b > a	(21.335)
				b > c	(6.982)
Phosphorus	1.00*	0.96	0.89	a > c	(4.028)
Sodium	0.61	0.84*	0.63	b > a	(6.156)
				b > c	(4.976)
Uranium	0.55	0.82*	0.50	b > a	(8.094)
				b > c	(9.913)
Zinc	0.68	0.87*	0.76	b > a	(4.457)

*Critical value for G-test: $\chi^2_{(df = 1)} = 3.841$

Table 3.2 Frequencies of occurrence of selected elements in crystalline particulate matter and amorphous particulate matter from treatments without cells. Asterisks indicate significant differences.

Element	Crystalline particulate matter (a) (n = 18)	Amorphous particulate matter (b) (n = 30)	Significant differences (G-test values in parentheses)*	
Aluminum	0.94*	0.70	a > b	(4.751)
Arsenic	0.44	0.53		
Calcium	1.00*	0.77	a > b	(7.017)
Chlorine	0.89*	0.40	a > b	(12.264)
Cobalt	0.61	0.33		
Copper	0.39	0.20		
Iron	0.44	0.37		
Magnesium	0.61	0.47		
Manganese	0.72	0.53		
Molybdenum	0.83	0.80		
Nickel	0.83	0.83		
Phosphorus	0.67	0.47		
Sodium	0.89	0.73		
Uranium	0.78	0.57		
Zinc	0.83	0.70		

*Critical value for G-test: $\chi^2_{(df = 1)} = 3.841$

Table 3.3 Frequencies of occurrence of selected elements in crystalline particulate matter from treatments with cells and crystalline particulate matter from treatments without cells. Asterisks indicate significant differences.

Element	Crystalline particulate matter from treatments with cells (a) (n = 45)	Crystalline particulate matter from treatments without cells (b) (n = 18)	Significant differences (G-test values in parentheses)*	
Aluminum	0.87	0.94		
Arsenic	0.60	0.44		
Calcium	0.98	1.00		
Chlorine	0.67	0.89		
Cobalt	0.58	0.61		
Copper	0.33	0.39		
Iron	0.87*	0.44	a > b	(11.327)
Magnesium	0.98*	0.61	a > b	(14.309)
Manganese	0.96*	0.72	a > b	(6.319)
Molybdenum	0.96	0.83		
Nickel	0.91	0.83		
Phosphorus	0.96*	0.67	a > b	(6.716)
Sodium	0.84	0.89		
Uranium	0.82	0.78		
Zinc	0.87	0.83		

*Critical value for G-test: $\chi^2_{(df = 1)} = 3.841$

Table 3.4 Frequencies of occurrence of selected elements in amorphous particulate matter from treatments with cells and amorphous particulate matter from treatments without cells. Asterisks indicate significant differences.

Element	Amorphous particulate matter from treatments with cells (a) (n = 38)	Amorphous particulate matter from treatments without cells (b) (n = 30)	Significant differences (G-test values in parentheses)*	
Aluminum	0.68	0.70		
Arsenic	0.26	0.53*	b > a	(5.211)
Calcium	0.87	0.77		
Chlorine	0.68*	0.40	a > b	(5.546)
Cobalt	0.37	0.33		
Copper	0.34	0.20		
Iron	0.39	0.37		
Magnesium	0.79*	0.47	a > b	(7.729)
Manganese	0.53	0.53		
Molybdenum	0.92	0.80		
Nickel	0.68	0.57		
Phosphorus	0.89*	0.47	a > b	(13.021)
Sodium	0.63	0.73		
Uranium	0.50	0.57		
Zinc	0.76	0.70		

*Critical value for G-test: $\chi^2_{(df = 1)} = 3.841$

3.3.2 X-PEEM spectroscopy results

A high resolution micrograph of a *C. noctigama* cell taken with the SPHINX X-PEEM spectromicroscope is shown in Figure 3.3. The cell is surrounded by the silicon wafer on which it is mounted (light area) and by debris (dark areas) that likely consists of dead or crumpled cells from the axenic culture from which the sample was taken. Division maps show uranium (Figure 3.4a) strongly co-localized with both carbon (Figure 3.4b) and phosphorus (Figure 3.4c) on the surface of the *C. noctigama* cell examined, particularly in the region around the cell body that appears to be a thin layer that may be cell membrane or exudate, but not surrounding the lower portions of the flagella. High concentrations of uranium can be seen in the indistinct regions to the left and to the right of the cell, again co-localized with carbon and phosphorus.

Spectra extracted from the cell are shown in Figure 3.5. Uranium occurs at 102 eV (Figure 3.5a), carbon as a set of three peaks at 286, 288 and 289 eV (Figure 3.5b), and phosphorus as a set of two close peaks at 135 and 136 eV (Figure 3.5c). Presumably each element is bound to other elements, likely including each other, but there are no reference spectra available for comparison, so the nature of the bonds between these elements remains unknown. Extensive searching for regions within the sample that exhibited different spectral signatures, with peaks occurring at different locations than in the spectra shown in Figure 3.5, for instance as would occur if there were a chemical interaction that was unique at a particular site on the sample, yielded nothing of interest. Additionally, there is no evidence that indicates the presence of a crystalline cell product that may bind metals extracellularly.

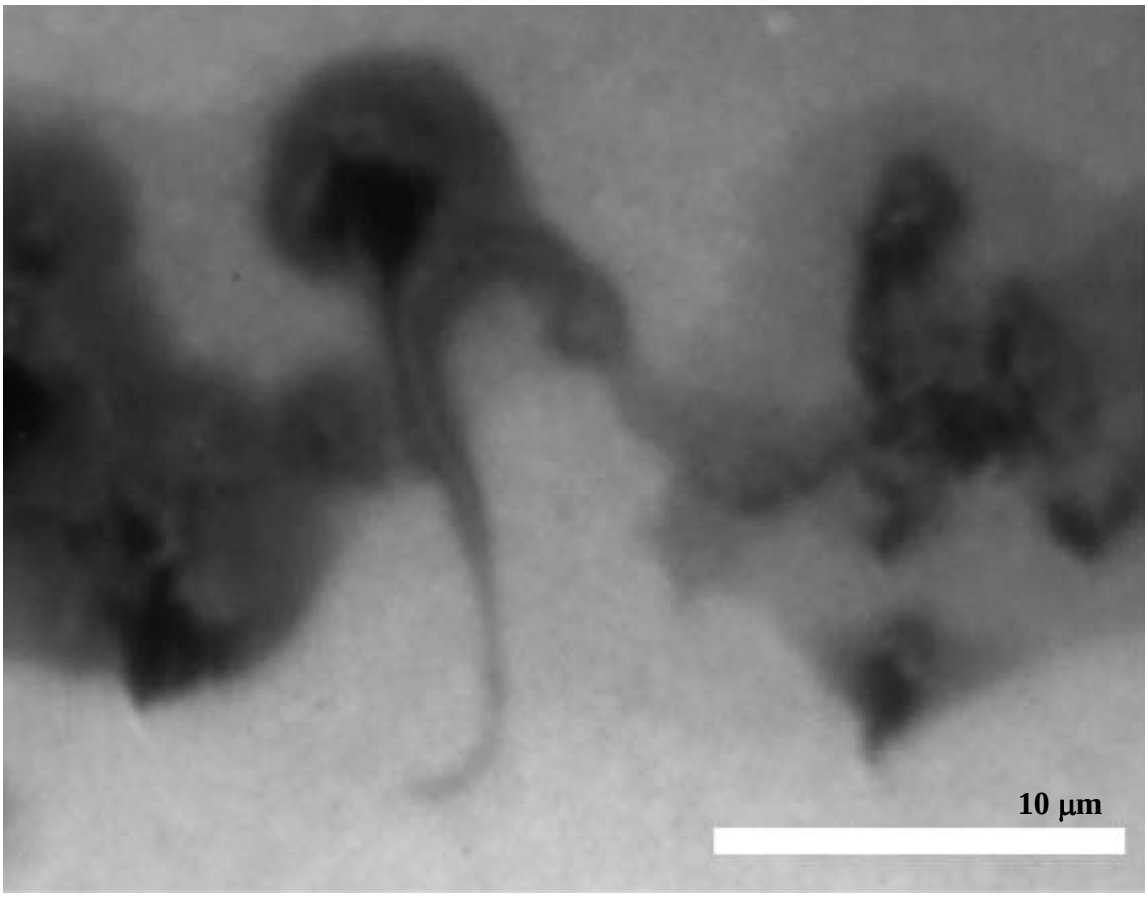


Figure 3.3 X-PEEM light micrograph of a *Chlamydomonas noctigama* cell.

Figure 3.4 Division maps showing the distribution of **a.** uranium (purple) **b.** carbon (teal), and **c.** phosphorus (yellow) in a *Chlamydomonas noctigama* cell. Dark regions indicate higher concentrations of each element. Uranium 102/96 eV; carbon: 289/283 eV; phosphorus 136/132 eV.

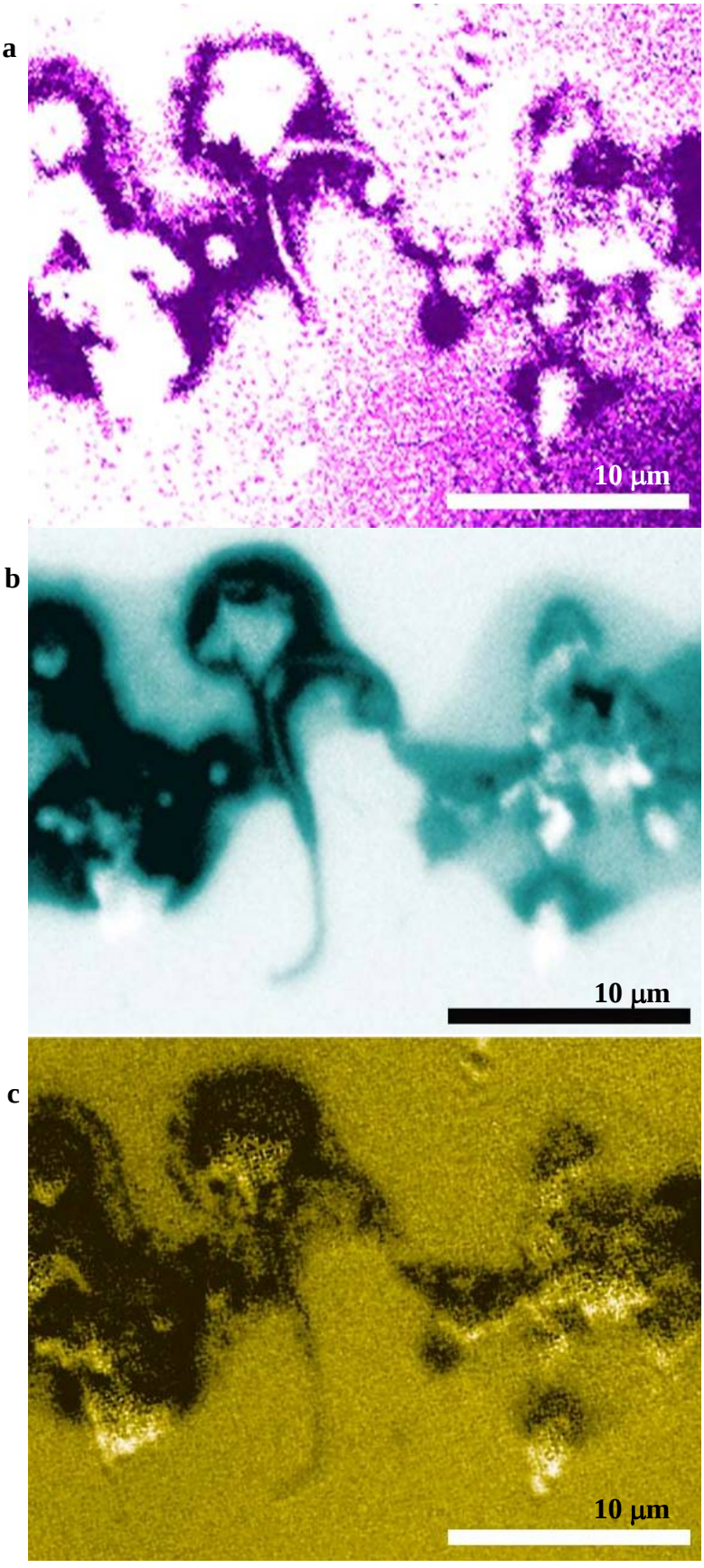
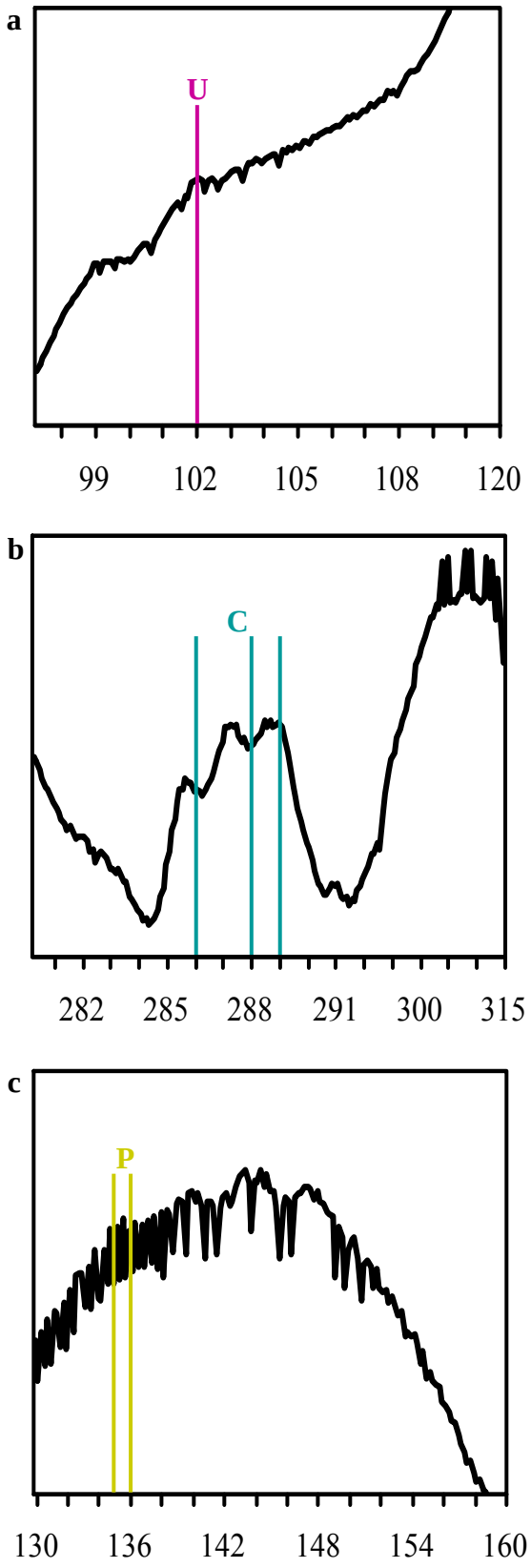

a
10 μm
b
10 μm
c
10 μm

Figure 3.5 X-PEEM spectra for uranium (a) carbon (b), and phosphorus (c) taken from the *Chlamydomonas noctigama* cell shown in Figure 3.3. Characteristic emission lines are shown for uranium at 102 eV, for carbon at 286, 287 and 289 eV, and for phosphorus at 135 and 136 eV.


a
U
99
102
105
108
120
b
C
282
285
288
291
300
315
c
P
130
136
142
148
154
160
Photon energy (eV)

3.4 Discussion

The experiments described in this chapter were attempts to discern where metals occurred in the particulate matter generated in the experiments described in Chapter 2, in treatments with and without *C. noctigama* cells. The results of the comparisons of spectra from EDX spectroscopy indicate that in the spectra from the treatments with cells, the bulk of the metals present occurred in crystalline particulate, followed by cells, with the smallest amount in the amorphous particulate (Figure 3.1). In spectra from the samples from the treatments without cells, more metals are present in crystalline particulate than in amorphous particulate. Spectra from each matter type (i.e. cells, crystalline particulate matter and amorphous particulate matter) were consistent between samples, without cells) so comparison by matter type between these spectra is quite strong. Comparison between crystalline and amorphous particulate matter from the samples with and without cells indicates greater concentrations of metals in crystalline particulate from the samples with cells. The X-PEEM spectroscopic data is limited to spectra from a single cell (the only data available), and so is without replication. Consequently, these data may not indicate what occurs in most cells.

Based on this assessment of the spectral data from EDX and X-PEEM spectroscopy, the metals of interest do seem to have a particular distribution in the various types of matter. Arsenic, nickel and uranium are present at the highest concentrations in crystalline particulate matter, followed by in cells, and with the lowest concentrations occurring in amorphous particulate matter, as summarized in Table 3.5. The highest concentrations of arsenic were in crystalline particulate in the treatment with cells, but it was found as often in cells. In treatments without cells, arsenic did not occur significantly more often in crystalline particulate than in amorphous particulate. Nickel occurred most frequently and at the highest concentration in crystalline particulate and at the second highest concentration in cells. When no cells were present, there was no particular localization of nickel, and it occurred as often but at lower concentrations in both crystalline and amorphous particulate in treatments without cells. Uranium

Table 3.5. Summary of ranked relative concentrations and frequencies of occurrence from EDX spectral data for arsenic, nickel and uranium in cells, crystalline particulate and amorphous particulate matter, with 1 indicating the highest concentration or frequency of occurrence in treatments with and without cells.

		Cells			No cells	
Metal	Spectral data inference	Cell	Crystalline Particulate	Amorphous Particulate	Crystalline Particulate	Amorphous Particulate
As	Concentration	2	1	3	1	2
	Frequency	1.5	1.5	3	1.5	1.5
Ni	Concentration	2	1	3	1	2
	Frequency	2.5	1	2.5	1.5	1.5
U	Concentration	2	1	3	1	2
	Frequency	2.5	1	2.5	1.5	1.5

occurred most frequently and at the highest concentrations in crystalline particulate in the cell treatment, but also occurred at high concentrations in association with cells.

There does not seem to be any literature describing sorption of arsenic to the exterior of cells, but Kobayashi *et al.* (2005) describe algal uptake of arsenate by phosphate uptake pathways occurring as a result of similarities in coordination chemistry of arsenates and phosphates. In my experiments, arsenic occurred at high concentrations, and it is possible that arsenic was removed from the water not only by complexation with other elements as a component of the crystalline particulate matter, but also directly, by the cell. However, arsenic was not observed at specific subcellular locations to provide evidence for this speculation, and I do not know what form the arsenic in solution or in association with particulate matter took.

Nickel is the only one of the three metals of interest that has a role in the healthy functioning of cells (at very low levels), as a component of the enzyme urease, and cell uptake pathways for nickel occur in algae (Mulrooney and Hausinger 2003; Wang and Wood 1984; Watt and Ludden 1999). There is no evidence that the cells used in these experiments used passive sorption or active uptake to remove nickel from solution, but it is likely that both mechanisms were active. It is also likely that, relatively speaking, more passive sorption occurred, due to extremely high concentrations of nickel in the DJX water, and simply because passive sorption occurs independently of any cell-mediated response to its environment. Wall sorption of nickel has been observed by Deng *et al.* (2003). Mehta and Gaur (1999) documented sorption of nickel by extracellular chelation with proline, which might be the means by which crystalline particulate forms. However, there are numerous instances of cells producing extracellular metal-chelating proteins and polypeptides (Sirinipornadulsil 2002; Chan *et al.* 2004; Dreschel and Jung 1998; Howe and Merchant 1992; Leal *et al.* 1999; Lee *et al.* 1996; Mehta and Gaur 1999; Ahner and Morel 1995; Bruland *et al.* 1991), and so neither is this an unlikely scenario.

The association of uranium with cells is supported by the X-PEEM mapping and spectroscopy, where carbon, phosphorus and uranium were strongly co-localized on the cell and in the region immediately surrounding it. The X-PEEM map of uranium occurring in association with *C. noctigama* provides clear evidence that this alga

removes uranium from water, in contradiction with Suzuki *et al.* (2005), who found no evidence of any algal-uranium association in uranium mine sediments. Their result may be a product of metal-cell dissociation in reducing conditions, and thus there may not be a relevant basis for comparison. However, my result, that the alga removed uranium from water, is in agreement with results of numerous other researchers (Adair and Apt 1990; Fowle *et al.* 2000; Kelly *et al.* 2001; Mann and Fyfe 1985; Renninger *et al.* 2004). It is likely that the bulk of the uranium attached to the *C. noctigama* cell got there by passive sorption. It is possible that some uranium is taken up by the alga, which must then transport it out of the cell by some mechanisms and deposit it to the exterior (Backor *et al.* 2003; Fortin 2004; Langley and Beveridge 1999; Mann and Fyfe 1985; Merroun *et al.* 2005; Renninger *et al.* 2004), or that the cell is induced to produce extracellular chelators which remove the metal from solution (Chan *et al.* 2004; Dreschel and Jung 1998; Howe and Merchant 1992; Leal *et al.* 1999; Mehta and Gaur 1999; Mejare and Bulow 2001), as may also be the case for nickel. Because such induction does not seem to be specific to any particular metal or concentration of metals, but rather occurs with many metals at many, including extremely low concentrations (Ahner and Morel 1995; Bruland *et al.* 1991), this is not an unlikely mechanism by which crystalline particulate matter may form in the presence of cells. This is, however, a speculative conclusion since I have no evidence that *C. noctigama* induced the production of the metal-chelating crystalline particulate matter that seemed to occur at a greater rate (Chapter 2) in the presence of cells than in the absence of them.

Phosphorus, the other element with which we are concerned, occurred at a higher frequency in cells than in amorphous particulate matter in treatments with cells (Table 3.1). Since phosphorus is a necessary component of many biological molecules, this is not surprising. The lack of a significant difference between its occurrence in crystalline and amorphous particulate matter (Table 3.1 and Table 3.2) is interesting, however, because it indicates that the localization of phosphorus does not co-vary with the elements of interest. That is, phosphorus may not directly induce the sedimentation of arsenic, nickel or uranium. However, its significantly higher presence in crystalline particulate matter from treatments with cells compared to treatments without cells

indicates that *C. noctigama* induces the production of a crystalline particulate matter with a higher phosphorus concentration than would be formed abiotically under the same conditions. That this phenomenon coincides with the increased presence of metals in the crystalline particulate matter is just that: coincidence.

Finally, the crystalline particulate matter formed in the presence of cells may be hydroxyapatite. The spectra from this matter shosw a signature that is characteristic of hydroxyapatite (Bonli pers. comm. 2006). Biogenic hydroxyapatite ($Ca_5(PO_4,CO_3)_3(OH)$ is also known as dahllite (Weiner and Dove 2003), and sorbs various metals and metalloids, including arsenic and uranium (Czerniczyniec *et al.* 2007). There seems to be no literature on the subject of chlorophyte algae and biogenic minerals, but numerous bacteria (Bazylinski and Frankel 2003; Chan *et al.* 2004; Fortin 2004) and some algal groups produce diverse biogenic minerals (Perry 2003; Young and Henrickson 2003). It may be a short road from secreting metal-chelating cell products into the extracellular milieu to the templation of regular mineral structures. In fact, much templation of biogenic minerals occurs in this manner.

CHAPTER 4. INVESTIGATION INTO THE UTILITY OF LUGOL'S IODINE AS A PRESERVATIVE FOR TRACE METALS ANALYSIS USING SPECTROSCOPIC METHODS

4.1 Introduction

A field-ready method of preserving algae and bacteria for the identification of trace metals using spectromicroscopy would be very useful, and it is worth investigating the utility of the classic preservative Lugol's iodine for this application. Lugol's-preserved cells maintain excellent morphological detail, it is a simple, field-ready method of preservation, and there are abundant historical samples preserved with Lugol's iodine that could be analyzed spectroscopically. A literature search revealed no previous studies on the topic of the utility of Lugol's iodine-preserved cells for spectromicroscopy.

Lugol's iodine is commonly used to kill and stain cells for identification, counting and long-term storage (Lund, Kipling and Le Cren 1958; Woelfl and Whitton 2000). Lugol's iodine is prepared by combining potassium iodide with sublimed iodine and sodium acetate[6]. Use of Lugol's iodine is simple: a small amount is added to a water sample with cells in it, and allowed to rest until the cells drop to the bottom of the container. As a dye, Lugol's iodine provides excellent contrast for viewing fine cellular detail in light, phase contrast and dark-field microscopy. It has been used in histology as a differential stain to dye cellulose blue, chitin reddish-violet, amyloid brown and glycogen red-brown (Lillie 1977). *Chlamydomonas* stains brown.

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[6] Lugol's iodine recipe: Combine and stir till dissolved 10 g potassium iodide (KI) in 20 mL water, 5 g sublimed iodine (I_2) in 50 mL water and 5 g sodium acetate ($NaCH_3CO_2$). Store in brown glass bottle capped with plastic cap with a polyvinyl insert. Use 0.5 to 1 mL Lugol's iodine per 100 mL sample.

Killing occurs rapidly when iodine penetrates the cell wall. The reactions andreaction products are not known (Gottardi 2001), but it is thought that there are three ways that iodine kills cells. The first is by oxidation of sulfhydryl groups of cysteine, resulting in the loss of the ability to form disulfide bridges, thereby disrupting protein folding and activity. Second, iodination of the phenolic and imidazolic groups of tyrosine and histidine increase the size of molecules these amino acids are in, possibly resulting in steric hindrance and preventing formation of required hydrogen bonds in molecules such as DNA. Third, the interaction of iodine with unsaturated fatty acids may lead to changes in the physical properties of lipids resulting in membrane immobilization and breakage (Gottardi 2001). Some of these changes may affect cell-metal interactions and subcellular metal localization.

My objective for this chapter is to determine if algal samples preserved by Lugol's iodine can be used to assess trace metals by spectromicroscopy.

4.2 Methods

Cell cultures used in this experiment were grown at the same time and in the same manner as the second metal uptake experiment as described in Chapter 2. Two replicate flasks of the same treatment, *Chlamydomonas noctigama* grown in DJX water with no phosphorus added, were used. At experiment end, 2 mL Lugol's iodine was added to each flask. The flasks were capped and set aside to allow time for the Lugol's iodine to act on the cells and to permit sedimentation of particulate matter to the bottom of the flasks. After one week, each flask was gently stirred to re-suspend the particulate matter within, which was then filtered through 0.1 μm filters. Filters with filtered particulate matter were placed in a dessicator for 48 hours. Particulate matter included cells, crystalline and amorphous particulate matter. Samples were prepared for EDX spectroscopy and scanned by the method described in Chapter 3. Spectra from cells, crystalline and amorphous particulate matter were obtained. Spectra from samples treated with Lugol's iodine were compared to the EDX spectra from samples with cells that were not treated with Lugol's iodine, from both experiment 1 and experiment 2,

using the methodology described in Chapter 3, to show if the distribution of metals and other elements in samples is affected by treatment with Lugol's iodine.

4.3 Results

EDX spectra from cells, crystalline particulate and amorphous particulate matter preserved with Lugol's iodine (Figure 4.1) demonstrate great similarity between these three matter types, including elements present in spectra and relative peak heights. The peak strengths for arsenic, nickel and uranium in both cells and particulate matter are less in Lugol's iodine preserved samples than in unpreserved samples (Figures 4.2 and 4.3). In fact, the dominant feature of all the spectra from samples treated with Lugol's iodine is the strong presence of iodine, suggesting that many of the elements present in unpreserved samples are present in decreased concentrations in samples preserved by Lugol's iodine.

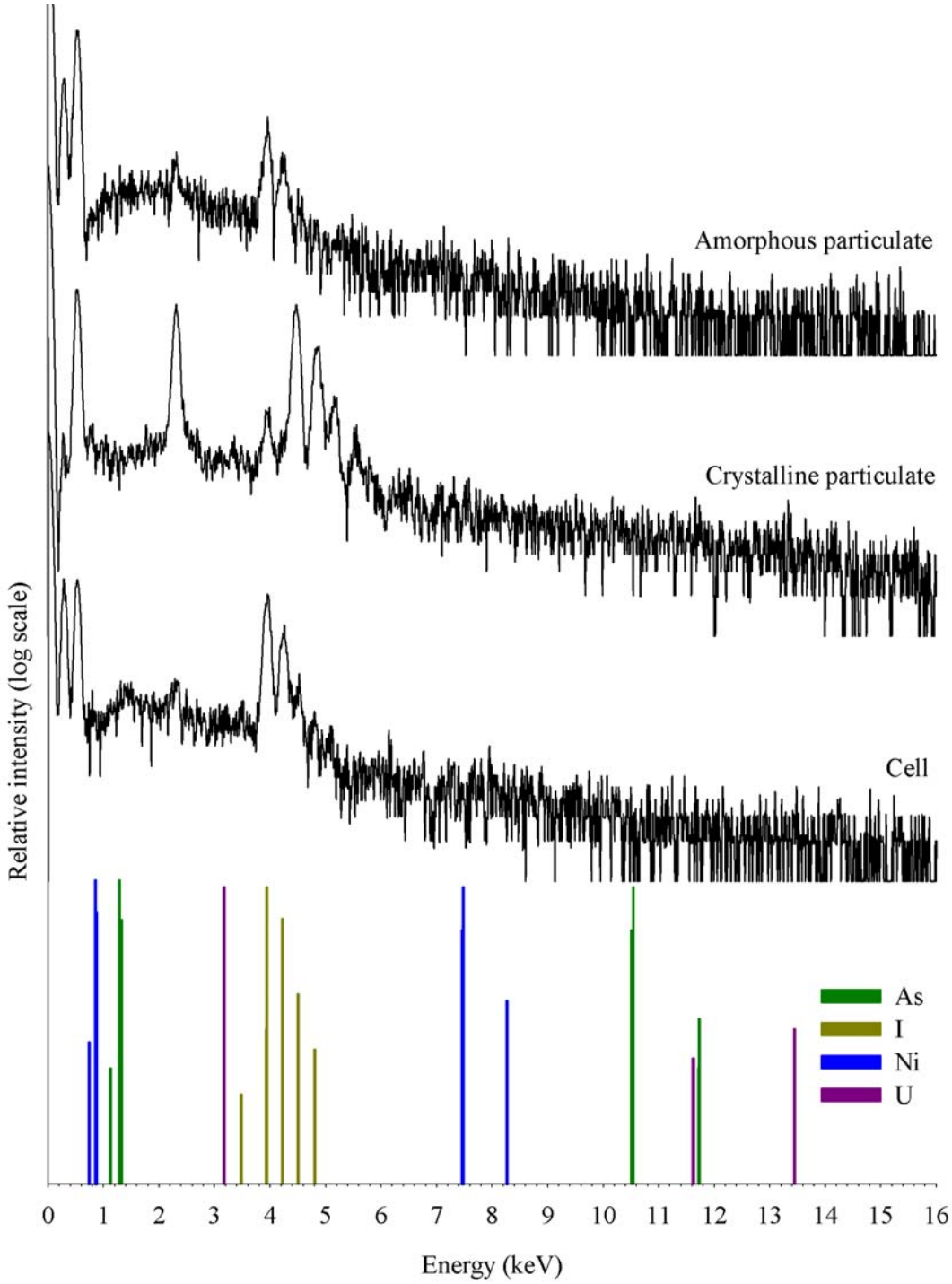

Figure 4.1 EDX spectra from a cell and from crystalline and amorphous particulate matter preserved with Lugol's iodine, showing the relative intensities (log scale) of contributing elements over the range of energies from 0 to 16 keV. Characteristic relative intensities of emission lines for the elements arsenic, iodine, nickel and uranium in this range are shown. Spectra are offset on the Y-axis to facilitate comparison.

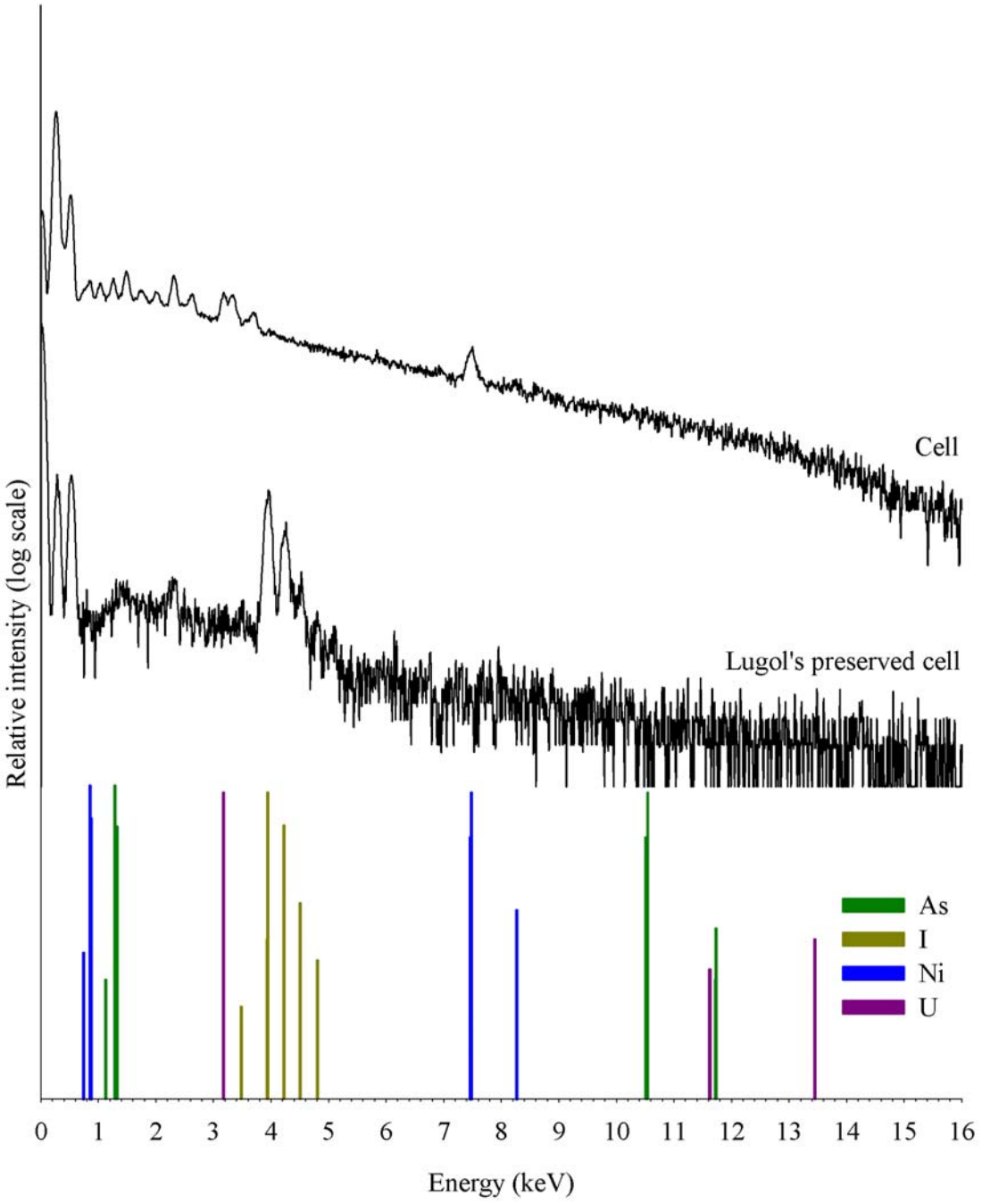

Figure 4.2 EDX spectra from a cell preserved with Lugol's iodine and from a non-preserved cell, showing the relative intensities (log scale) of contributing elements over the range of energies from 0 to 16 keV. Characteristic relative intensities of emission lines for the elements arsenic, iodine, nickel and uranium in this range are shown. Spectra are offset on the Y-axis to facilitate comparison.

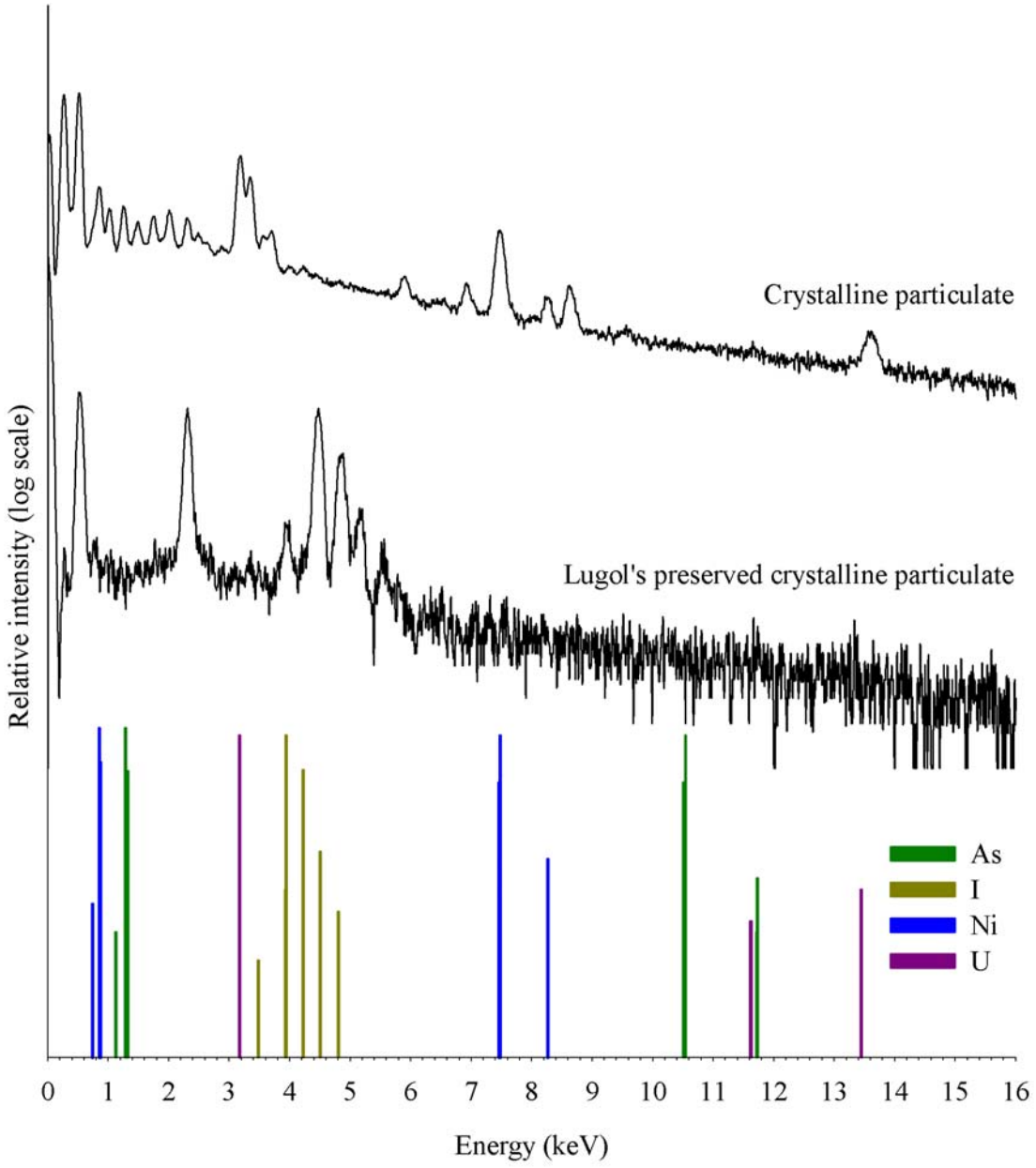

Figure 4.3 EDX spectra from crystalline particulate matter preserved with Lugol's iodine and from non-preserved crystalline particulate matter, showing the relative intensities (log scale) of contributing elements over the range of energies from 0 to 16 keV. Characteristic relative intensities of emission lines for the elements arsenic, iodine, nickel and uranium in this range are shown. Spectra are offset on the Y-axis to facilitate comparison.

4.4 Discussion

It is clear, based on comparison of spectra from samples treated with Lugol's iodine and unpreserved samples, that Lugol's iodine induces significant changes in the distribution of elements in samples. There was increased homogeneity in Lugol's preserved samples relative to unpreserved samples, characterized by decreased partitioning of individual elements in crystalline particulate and in cells in samples preserved by Lugol's iodine. Additionally, the higher concentrations of metals in Lugol's preserved amorphous particulate matter relative to those in unpreserved amorphous particulate matter may indicate that metals sorbed to crystalline particulate and to cells are somehow removed from their substrate by Lugol's iodine. In the case of cells, this might possibly be the result of iodine-induced cell damage such as membrane breakage (Gottardi 2001).

In conclusion, Lugol's iodine does not adequately preserve samples for analysis of trace metals by spectroscopic methods such as EDX. It is my recommendation that those interested in understanding the partitioning of elements between matter types in aquatic samples or who are interested in the subcellular localization of elements use unpreserved samples, or investigate other preservatives whose character may not modify the nature of the sample. It is not likely that archived samples preserved with Lugol's iodine would be usefully analyzed using spectroscopic methods.

CHAPTER 5 SYNTHESIS

5.1 Summary and synthesis of results

The general results of Chapters 2 and 3 are summarized in Figure 5.1.

5.1.1 Biotic removal of arsenic, nickel and uranium

Results of the experiments described in Chapter 2 indicate that removal of arsenic, nickel and uranium from water from the DJX pit at Cluff Lake uranium mine is enhanced by the presence of the chlorophyte alga *C. noctigama*. The general trend was for metals to be present in greater concentrations in particulate matter from treatments with cells than from treatments without cells (Figure 5.1). There was a corresponding decrease in dissolved metal concentrations. These results have a high degree of certainty, because they were repeated for both particulate and dissolved metal concentrations in three replicates of each treatment in experiment 1 and five in experiment 2. Moreover, concentrations were measured using ICP-MS, which is a highly accurate and standard technique for trace metals analysis.

The degree to which *C. noctigama* removed arsenic, nickel and uranium from the water and the corresponding increase of these metals in particulate matter (Table 5.1) demonstrates that sedimentation was mainly biotically induced. This confirms speculation by Dessouki *et al*. (2005) that significant decreases in concentrations of metals in the surface water of the DJX pit and corresponding increases in sediment traps were biologically mediated. In Dessouki's experiments, phosphorus additions resulted in increased algal biomass, and decreases in surface metal concentrations were thought to be the result of these increases.

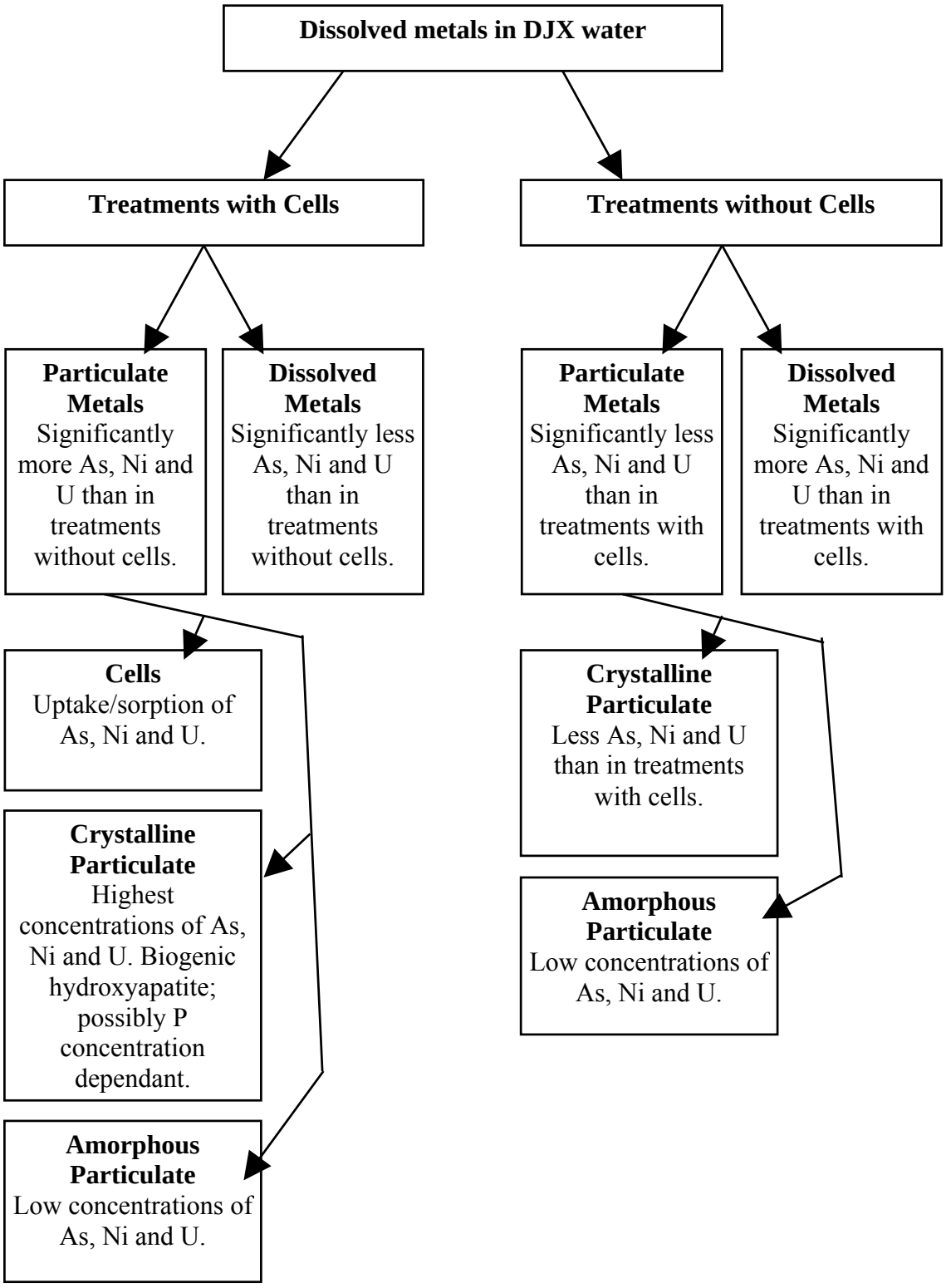

Figure 5.1 Flow diagram summarizing major trends and the partitioning of metals of interest in matter in the experiments described in Chapters 2 and 3.

Table 5.1 Percent decrease in dissolved arsenic, nickel and uranium concentrations from experiments 1 and 2, and from Dessouki's (2005) 2004 experiments.

	Expt. 1	Expt. 2	Dessouki (2005)
Arsenic	14 %	11.6 %	21 %
Nickel	15.8 %	5 %	27 %
Uranium	7 %	2.4 %	13 %

Overall, less metal was removed in my experiments that used a single alga than was observed in Dessouki's experiments. In his mesocosms, the entire microbial community present in the DJX pit is likely to have contributed to the observed declines of surface metal concentrations. This may indicate that a more complex microbial community that includes a variety of bacterial and algal species would increase the net removal of metals. *Chlamydomonas* spp. dominated the surface-water microbial communities in Dessouki's mesocosms, and in his sediment traps, the most common organism was a thick-walled unidentified Chlorophyte alga; it is possible that this organism was the encysted form of *Chlamydomonas*.

The small scale method that I used in this study was ideal for the determination of relative metal concentrations in particulate and dissolved matter, facilitated by the very accurate measurements of concentrations by ICP-MS. A larger-scale mesocosm approach would not likely improve accuracy. Additionally, potential microbial community dynamics are eliminated using an axenic culture approach, and there is no confusion about which organisms are present. The small-scale approach was dramatically more useful than a mesocosm-scale approach in terms of the mechanistic inferences that could be made from the results. For this reason, I recommend that researchers who are interested in investigating metal removal by more complete communities of microorganisms use the microcosm-scale approach.

5.1.2 Direct complexation of arsenic, nickel and uranium to *C. noctigama* cells

Direct complexation of arsenic, nickel and uranium to *C. noctigama* cells was observed by EDX spectroscopy. Arsenic, the metal which was present at the lowest concentration in the DJX water, was present on scanned cells in low concentrations, and nickel and uranium, which were present at high concentrations in the DJX water, were present at higher concentrations. All three metals occurred more frequently in association with crystalline particulate matter than in cells. This comparison is drawn from spectra from 44 cells and 45 pieces of crystalline particulate matter from all phosphorus treatment levels from both experiments, so the comparison is strong.

Examination of a single cell using X-PEEM spectromicroscopy showed uranium co-localized with carbon and phosphorus on the exterior of the cell which suggests that uranium bound to the extracellular region of the cell; that is, to (either or both of) the outside of the cell wall and an exopolysaccharide layer outside of the cell wall. That there was only one replicate reduces the strength of inference that can be made from these data. Spectra from the cell exhibited a uniform profile, which would seem to indicate that there was no particular region of the cell that was involved with catalysis of unique reactions, for instance at a particular wall moietie. The X-PEEM instrument operator's reluctance to work with uranium prevented the gathering of reference spectra from several uranium samples with different speciation, so no comparison to reference spectra could be made to identify the nature of the carbon-uranium-phosphorus bond that likely occurred in the sample.

Information obtained using X-PEEM spectromicroscopy was not adequate to characterize the nature of the cell-metal associations of interest. Numerous sample preparations were examined using X-PEEM. The most informative preparation was unpreserved and not fixed in any immobilizing medium, as described in Chapter 3. Preserved samples tended to not exhibit any discernable peaks that would indicate the presence of the element of interest over the range at which it was scanned. This problem was compounded by the reality that the best X-PEEM spectra are obtained from perfectly flat samples because uneven surfaces cause scattering of incident radiation which interferes with the quality of the resultant spectra. Attempts to embed cells in resin for sectioning yielded spectra that were devoid of interesting peaks, and which were indistinguishable from spectra from regions surrounding the embedded samples. I would recommend that researchers interested in exploring the nature of cell-metal interactions using X-PEEM spectromicroscopy limit their investigations to a single metal of interest. Before any progress can be made with this analytical technique, however, sample preparation issues must be resolved. Other synchrotron spectroscopic methods might be combined usefully with (or substituted for) X-PEEM.

The mechanisms by which arsenic, nickel and uranium were removed by *C. noctigama* remain uncertain, partially because of low replication of data and limited success with the analytical techniques chosen, as described above. Most metals,

including nickel and uranium also occurred adsorbed to cells, but at lower concentrations than in crystalline particulate matter. These results suggest two mechanisms by which *C. noctigama* removed arsenic, nickel and uranium from solution: by sorption to some cell product, and by direct sorption to the exterior of the cell. EDX spectroscopy techniques, as described in Chapter 3, could be well-employed in the determination of partitioning of metals between cells and other particulate matter. It would also be interesting to have an estimate of the concentrations of each metal in each type of matter.

5.1.3 Abiotic precipitation of arsenic, nickel and uranium

The contribution of abiotic precipitation in the sedimentation of metals was unclear from Dessouki's experiments. In my experiments, examination of particulate matter on filters with pore size 0.1 μm by SEM revealed the presence of crystalline particulate matter, largely occurring in the presence of cells, the formation of which may have been enhanced by a cell product. EDX spectroscopy suggested that this particulate matter was hydroxyapatite, a not uncommon cell product, and that this hydroxyapatite contained various metals, including arsenic, nickel and uranium. This mineral was present to a lesser degree in samples without cells, and the amount and frequency of metals contained in it was less than in samples with cells. It suggests that *C. noctigama* induces the production of biogenic hydroxyapatite which sequesters metals from the environment. However, the confidence in this conclusion is lower than the conclusion that *C. noctigama* removes metal from water. Although the methodology used is considered to be sound, all samples were drawn from only one replicate of each treatment from one experiment. Positive determination of the relative amounts of crystalline particulate matter in treatments with and without cells would be easily achievable with replication, using the SEM survey methods described in Chapter 2. Further exploration of the nature of the crystalline particulater matter may be warranted.

X-PEEM spectromicroscopy data from crystalline particulate matter was uninformative.

5.1.4 Dependence of metal removal on phosphorus concentration

I did not find any correlation between metal removal and phosphorus concentration in the media, or between algal density (equated simply to particulate mass in these experiments) and phosphorus concentration. These results contradict those of Dessouki *et al.* (2005). In his 2003 experiments, the removal of arsenic, cobalt, copper, manganese, nickel, uranium and zinc from the surface water to the sediments increased with phosphorus concentration. The difference is likely because phosphorus was not a growth-limiting nutrient in my experiments. I obtained water from the DJX pit in the late summer of 2004. Personnel at AREVA, the lease-holder and operator of the (former) Cluff Lake Mine, added a large amount of phosphorus to the water of the DJX pit in the fall of 2003 after witnessing the results of Dessouki's 2003 experiments, which had demonstrated the potential for remediation of mine water by phosphorus addition. Dessouki's 2004 experiments in the DJX pit, which were intended to increase replication of his data set and refine the quality of his results, produced more variable results than his 2003 experiments (Dessouki 2005); Dessouki posited that this was due to the greater phosphorus concentration compared to the previous year (Dessouki pers. comm. 2004).

There was, however, one indication of the role of phosphorus in the removal of metals from water. Production of crystalline particulate matter in cell cultures seemed to increase with phosphorus concentration. Unfortunately there was only one replicate of each treatment and so the result needs to be confirmed. A simple experiment with multiple replicates of each phosphorus concentration treatment and using SEM to estimate the amounts of crystalline particulate, as described in Chapter 2, would likely resolve this question.

5.1.5 Lugol's iodine as a preservative for trace metals spectroscopy

Comparison of EDX spectra from samples preserved in Lugol's iodine were significantly different than from unpreserved samples, with decreased concentrations of many elements, including metallic elements. Lugol's iodine is not a suitable preservative for trace metals spectroscopy.

5.2 Conclusions

1. Metal removal from water is significantly enhanced by the presence of *C. noctigama.*
2. A laboratory-based approach that uses ICP-MS to measure trace metal concentrations can be used to obtain accurate and informative results with regard to microorganismal metals uptake.
3. The presence of a complex microbial community may enhance metal removal from water compared to removal by a single species.
4. *C. noctigama* may have removed arsenic, nickel and uranium from the DJX water by complexation of metals to cell walls, and to crystalline particulate matter, the formation of which may be enhanced by a cell product.
5. The crystalline particulate matter contained higher concentrations of arsenic, nickel and uranium than cells did.
6. The crystalline particulate matter may have been hydroxyapatite.
7. The amount of crystalline precipitate formed may be dependant on the phosphorus concentration of the media.
8. EDX spectroscopy was informative with regard to metals partitioning between types of particulate matter, but did not provide quantitative concentration data.
9. X-PEEM spectromicroscopic techniques are promising but sample preparation methods must be refined to obtain quality results.
10. Lugol's iodine is not suitable as a preservative for trace metals spectroscopy.

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APPENDIX A
Summary of microbial metal sorption data from literature

Table A-1 Summary of microbial metal sorption data from literature.

Metal	Experimental organism	Conc. in media (mg/L)	Exposure time	Concentration in live cells (mg/g dry cells, or as noted)	Concentration in dead cells (mg/g dry cells, or as noted)	Author(s)
Total uptake						
Ag	*Chlamydomonas reinhardtii*	$1.1*10^{-3}$	1 h	3.02 mg/m^2	—	Fortin and Campbell (2001)[1]
As	Mixed algae and bacteria	0.005	29 days	—	1 mg/m^2	Dessouki (2005)[1]
As	*C. reinhardtii*	74.9	2 days	$2*10^{-4}$	—	Kaise et al. (1999)[2]
As	*C. reinhardtii*	4	20 min	—	58.3	Mahan et al. (1989)[1]
As	*Chlorella pyrenoidosa*	4	20 min	—	73.3	Mahan et al. (1989)[1]
As	*Chlorella vulgaris*	100	1 week	0.16	—	Murray et al. (2003)[1]
As	*C. vulgaris*	1000	1 week	2.74	—	Murray et al. (2003)[1]
Cd	Mixed algae	$4.5*10^{-3}$	L. term	$1.2*10^{-3}$	—	Gupta (1996)[1]
Cd	*C. reinhardtii*	337230	25 min	$2.8*10^{-6}$ mg/cm^2	—	Kola and Wilkinson. (2005)
Cd	*Sargassum fluitans*	> 700	3 h	—	125	Leusch et al. (1995)[3]
Cd	*C. reinhardtii*	1.12	24 h	5.7	—	Macfie and Welbourn (2000)[1]
Cd	*C. reinhardtii*	4	20 min	—	575	Mahan et al. (1989)[1]
Cd	*C. pyrenoidosa*	4	20 min	—	716.7	Mahan et al. (1989)[1]
Co	*C. vulgaris*	2.8	21 days	4	—	Coleman et al. (1971)[1]
Co	*C. reinhardtii*	0.884	24 h	0.9	—	Macfie and Welbourn (2000)[1]
Cu	Mixed algae	$2.5*10^{-2}$	L. term	0.113	—	Gupta (1996)[1]
Cu	*C. reinhardtii*	0.0635	40 min	$3.2*10^{-9}$ mg/g *Chl*	—	Hill et al. (1996)[1]
Cu	*Scenedesmus subspicatus*	63.54	L. term	0.6	—	Knauer et al. (1997)[1]
Cu	*S. fluitans*	>800	3 h	—	90	Leusch et al. (1995)[3]
Cu	*C. reinhardtii*	0.127	24 h	6.4	—	Macfie and Welbourn (2000)[1]
Mo	*C. reinhardtii*	4	20 min	—	165.8	Mahan et al. (1989)[1]
Mo	*C. pyrenoidosa*	4	20 min	—	185	Mahan et al. (1989)[1]

Table A-1 Summary of microbial metal sorption data from literature (*continued*).

Metal	Experimental organism	Conc. in media (mg/L)	Exposure time	Concentration in live cells (mg/g dry cells, or as noted)	Concentration in dead cells (mg/g dry cells, or as noted)	Author(s)
Total uptake *continued*						
Mn	Mixed algae	0.2045	L. term	0.658	—	Gupta (1996)[1]
Ni	*Chlorella sorokiniana*	25	2 h	—	22	Akhtar et al. (2004)
Ni	*Escherichia coli*	0.08	55 min	1.5	—	Deng et al. (2003)[1]
Ni	Mixed algae and bacteria	0.970	29 days	—	200 mg/m^2	Dessouki (2005)[1]
Ni	*S. fluitans*	>800	3 h	—	75	Leusch et al. (1995)[3]
Ni	*C. reinhardtii*	0.440	24 h	0.4	—	Macfie and Welbourn (2000)[1]
Ni	*C. reinhardtii*	4	20 min	—	85	Mahan et al. (1989)[1,4]
Ni	*C. pyrenoidosa*	4	20 min	—	133.3	Mahan et al. (1989)[1]
Ni	*Pseudokirchneriella subcapitata*	2	3 days	450 mg/L	—	Mann and Fyfe (1984)[5]
Ni	*Ankistrodesmus* sp.	2	3 days	330 mg/L	—	Mann and Fyfe (1984)
Ni	*P.subcapitata*	2	22 days	55 mg/L	—	Mann and Fyfe (1984)[5]
Ni	*Ankistrodesmus* sp.	2	16 days	360 mg/L	—	Mann and Fyfe (1984)
Ni	*C. regularis*	58.7	1 h	0.13	0.14	Nakajima et al. (1981)[1,3]
Ni	*Synechococcus ATCC 17146*	$2.0*10^{-5}$	6 h	$4.2*10^2$	—	Wang and Wood (1984)[1]
Ni	*Oscillatoria UTEX 1270*	$2.0*10^{-5}$	6 h	$9.5*10^2$	—	Wang and Wood (1984)[1]
Ni	*Scenedesmus ATCC 11460*	$2.0*10^{-5}$	6 h	$7.9*10^2$	—	Wang and Wood (1984)[1]
Ni	*Chlamydomonas UTEX89*	$2.0*10^{-5}$	6 h	$5.4*10^2$	—	Wang and Wood (1984)[1]
Pb	Mixed algae	0.0273	L. term	0.026	—	Gupta (1996)[1]
Pb	*S. fluitans*	> 400	3 h	—	320	Leusch et al. (1995)[3]
Zn	*C. vulgaris*	35	3 days	67	—	Coleman et al. (1971)[1]
Zn	Mixed algae	0.0376	L. term	0.109	—	Gupta (1996)[1]
Zn	*S. fluitans*	> 1100	3 h	—	60	Leusch et al. (1995)[3]

Table A-1 Summary of microbial metal sorption data from literature (*continued*).

Metal	Experimental organism	Conc. in media (mg/L)	Exposure time	Concentration in live cells (mg/g dry cells, or as noted)	Concentration in dead cells (mg/g dry cells, or as noted)	Author(s)
Total uptake *continued*						
U	Mixed algae and bacteria	2.233	29 days	—	500 mg/m^2	Dessouki (2005)[1]
U	*Ankistrodesmus* sp.	0.04	—	0.006 mg/L	—	Mann and Fyfe (1985)[1]
U	Thames River algae (mixed)	1.5*10^{-3}	L. term	28 mg/L	—	Mann and Fyfe (1985)[1]
U	Elliot Lake algae (mixed)	1-2	L. term	400 mg/L	—	Mann and Fyfe (1985)[1]
U	*P. subcapitata*[2]	2	3 days	8570 mg/L	—	Mann and Fyfe (1984)
U	*Ankistrodesmus* sp.	2	3 days	1310 mg/L	—	Mann and Fyfe (1984)
U	*P. subcapitata*[2]	2	22 days	9040 mg/L	—	Mann and Fyfe (1984)
U	*Ankistrodesmus* sp.	2	16 days	6770 mg/L	—	Mann and Fyfe (1984)
U	*C. regularis*	238	1 h	17	37	Nakajima et al. (1981)[1,3]
U	*Tricholoma conglobatum*	9.5*10^{-3}	1 h	60.2	—	Nakajima and Sakaguchi (1993)
Intracellular uptake						
As	*C. reinhardtii*	300	L. term	1.9*10^{-6} mg/g *Chl*	—	Kobayashi et al. (2005) [1]
Cd	*Thalassiosira weisflogii*	3.4*10^{-3}	22 h	5.73*10^{-12} mg/cell	—	Ahner and Morel (1995) [1]
Cd	*P. subcapitata*	0.0562	1 h	6.1*10^{-4} mg/m^2	—	Campbell et al. (2002) [1,5]
Cd	*P. subcapitata*	0.0686	30 min	2.6*10^{-3} mg/m^2	—	Errecalde and Campbell (2000)[1,5]
Cd	*C. reinhardtii*	0.0562	1 h	5.62*10^{16} mg/cm^2	—	Kola and Wilkinson (2005) [1]
Cd	*C. reinhardtii*	1.12	24 h	2.6	—	Macfie and Welbourn (2000) [1]
Cd	*Chlorella autotrophica*	2.0*10^{-4}	5 h	~8000	—	Wang and Dei (2001a) [1]
Cd	*C. autotrophica*	2.0*10^{-4}	5 h	~1200	—	Wang and Dei (2001a) [1]
Co	*C. reinhardtii*	0.884	24 h	0.3	—	Macfie and Welbourn (2000) [1]
Cu	*Anabaena variabilis*	4.45	L. term	5.67	—	Hashemi et al. (1994) [1]

Table A-1 Summary of microbial metal sorption data from literature (*continued*).

Metal	Experimental organism	Conc. in media (mg/L)	Exposure time	Concentration in live cells (mg/g dry cells, or as noted)	Concentration in dead cells (mg/g dry cells, or as noted)	Author(s)
Intracellular uptake *continued*						
Cu	*S. subspicatus*	63.55	L. term	6.35	—	Knauer et al. (1997) [1]
Cu	*C. reinhardtii*	0.127	24 h	4.8	—	Macfie and Welbourn (2000) [1]
Ni	*T. weisflogii*	$8.2*10^{-5}$	22 h	$1.76*10^{-11}$ mg/cell	—	Ahner and Morel (1995) [1]
Ni	*C. reinhardtii*	0.440	24 h	0.2	—	Macfie and Welbourn (2000) [1]
Ni	*C. vulgaris*	2.93	16 h	$1.06*10^{-11}$ mg/cell	—	Mehta and Gaur (1999) [1]
Se	*C. autotrophica*	$1.6*10^{-5}$	5 h	~120	—	Wang and Dei (2001a) [1]
Se	*C. autotrophica*	$1.6*10^{-5}$	5 h	~450	—	Wang and Dei (2001a) [1]
Zn	*T. weisflogii*	$9.8*10^{-5}$	22 h	$5.49*10^{-11}$ mg/cell	—	Ahner and Morel (1995) [1]
Zn	*Chlamydomonas variabilis*	1.0	10 m	0.196	—	Bates et al. (1982) [1]
Zn	*C. autotrophica*	$1.4*10^{-4}$	5 h	~22000	—	Wang and Dei (2001a) [1]
Extracellular uptake						
Cd	*C. reinhardtii*	1.12	24 h	3.2	—	Macfie and Welbourn (2000) [1]
Co	*C. reinhardtii*	0.884	24 h	0.6	—	Macfie and Welbourn (2000) [1]
Cu	*A. variabilis*	4.45	L. term	1.20	—	Hashemi et al. (1994) [1]
Cu	*S.subspicatus*	63.55	L. term	0.6	—	Knauer et al. (1997) [1]
Cu	*C. reinhardtii*	0.127	24 h	1.6	—	Macfie and Welbourn (2000) [1]
Ni	*C. reinhardtii*	0.440	24 h	0.2	—	Macfie and Welbourn (2000) [1]
U	*Bacillus subtilis*	20	2 h	13.3	—	Fowle et al. (2000) [1]
Zn	*C. variabilis*	1.0	10 min	0.75	—	Bates et al. (1982) [1]

Table A-1 Summary of microbial metal sorption data from literature (*continued*).

[1] For ease of comparison between different studies, diverse units have been converted to units of mg/L and mg/g. Where conversion was impossible, the numerator was converted and the denominator retains the original units.
[2] Not dry biomass.
[3] Dead biomass chemically modified by acid treatment.
[4] Heat killed, lyophilized biomass.
[5] The alga formerly known as *Selenastrum capricornutum* is now referred to as *Pseudokirchneriella subcapitata.* For the sake of clarity, the modern name *P. subcapitata* is used in this table.

APPENDIX B
Dissolved and particulate metal concentrations of DJX water

Table B-1 Mean concentrations of dissolved metals in DJX pit surface water filtered through 0.1μm pore size filters (n = 6), and concentrations of metals in the filter-collected particulate matter (n = 1). Standard deviation of means in parentheses. Metal concentrations measured by ICP-MS. Dissolved metals reported as μg/L, particulate metals reported as g/L. Data from a preliminary study on the DJX pit, June 2004.

Element	Dissolved metal concentration (μg/L)		Particulate metal concentration (g/L)
Aluminum	33.73	(8.93)	1048.70
Arsenic	2.55	(0.28)	3.57
Boron	1380.10	(81.5)	10.52
Barium	19.81	(0.62)	1.91
Cadmium	ud	—	0.007
Cobalt	244.60	(15.2)	0.74
Chromium	0.58	(0.13)	1.45
Copper	14.45	(0.99)	5.67
Iron	613.33	(88.95)	211.41
Lead	1.48	(0.28)	0.43
Magnesium	119437.40	(19901.73)	225.36
Molybdenum	705.10	(31.05)	4.42
Nickel	1146.03	(77.46)	4.06
Phosphorus	693.73	(195.47)	8.49
Selenium	5.49	(1.88)	Ud
Silicon	—	—	0.00
Silver	ud	—	0.022
Strontium	2280.00	(73.9)	2.79
Titanium	9.47	(0.52)	2.65
Uranium	2913.80	(77.72)	212.22
Vanadium	0.25	(0.04)	8.74
Zinc	174.94	(9.44)	6.18
Zircon	0.03	(0.03)	0.29

APPENDIX C
Energies and intensities of emission lines for selected elements

Table C-1 Photon energies and relative intensities of K-, L- and M-shell lines of selected elements, arranged by increasing energy over the range from 0 to 20000 eV. An intensity of 100 is assigned to the strongest line in each shell for each element. (Adapted from Thompson *et al.* (2001))

Energy (eV)	Element	Line	Relative intensity
277.0	C	$K\alpha_{1,2}$	147
524.9	O	$K\alpha_{1,2}$	151
556.3	Mn	L_1	15
637.4	Mn	$L\alpha_{1,2}$	111
648.8	Mn	$L\beta_1$	77
677.8	Co	L_1	10
705.0	Fe	$L\alpha_{1,2}$	111
718.5	Fe	$L\beta_1$	66
742.7	Ni	L_1	9
776.2	Co	$L\alpha_{1,2}$	111
791.4	Co	$L\beta_1$	76
811.1	Cu	L_1	8
851.5	Ni	$L\alpha_{1,2}$	111
868.8	Ni	$L\beta_1$	68
884	Zn	L_1	7
929.7	Cu	$L\alpha_{1,2}$	111
949.8	Cu	$L\beta_1$	65
1011.7	Zn	$L\alpha_{1,2}$	111
1034.7	Zn	$L\beta_1$	65
1041.0	Na	$K\alpha_{1,2}$	150
1120	As	L_1	6
1253.6	Mg	$K\alpha_{1,2}$	150
1282.0	As	$L\alpha_{1,2}$	111
1317.0	As	$L\beta_1$	60
1486.3	Al	$K\alpha_2$	50
1486.7	Al	$K\alpha_1$	100
1557.4	Al	$K\beta_1$	1
2012.7	P	$K\alpha_2$	50
2013.7	P	$K\alpha_1$	100
2015.7	Mo	L_1	5
2139.1	P	$K\beta_1$	3
2289.8	Mo	$L\alpha_2$	11
2293.2	Mo	$L\alpha_1$	100
2306.6	S	$K\alpha_2$	50
2307.8	S	$K\alpha_1$	100
2394.8	Mo	$L\beta_1$	53
2464.0	S	$K\beta_1$	5
2518.3	Mo	$L\beta_{2,15}$	5
2620.8	Cl	$K\alpha_2$	50
2622.4	Cl	$K\alpha_1$	100
2623.5	Mo	$L\gamma_1$	3
2815.6	Cl	$K\beta_1$	6
3170.8	U	$M\alpha_1$	100
3485.0	I	L_1	4
3311.1	K	$K\alpha_2$	50
3313.8	K	$K\alpha_1$	100

Table C-1 Energies and intensities of emission lines of selected elements (*continued*).

Energy (eV)	Element	Line	Relative intensity
3589.6	K	$K\beta_{1,3}$	11
3688.1	Ca	$K\alpha_2$	50
3691.7	Ca	$K\alpha_1$	100
3926.0	I	$L\alpha_2$	11
3937.6	I	$L\alpha_1$	100
4012.7	Ca	$K\beta_{1,3}$	13
4220.7	I	$L\beta_1$	61
4507.5	I	$L\beta_{2,15}$	19
4800.9	I	$L\gamma_1$	8
5887.6	Mn	$K\alpha_2$	50
5898.8	Mn	$K\alpha_1$	100
6390.8	Fe	$K\alpha_2$	50
6403.8	Fe	$K\alpha_1$	100
6490.4	Mn	$K\beta_{1,3}$	17
6915.3	Co	$K\alpha_2$	51
6930.3	Co	$K\alpha_1$	100
7058.0	Fe	$K\beta_{1,3}$	17
7460.9	Ni	$K\alpha_2$	51
7478.2	Ni	$K\alpha_1$	100
7649.4	Co	$K\beta_{1,3}$	17
8047.8	Cu	$K\alpha_1$	100
8264.7	Ni	$K\beta_{1,3}$	17
8615.8	Zn	$K\alpha_2$	51
8638.9	Zn	$K\alpha_1$	100
8905.3	Cu	$K\beta_{1,3}$	17
10508.0	As	$K\alpha_2$	51
10515.8	As	$K\alpha_1$	100
11618.3	U	L_1	7
11720.3	As	$K\beta_3$	6
11726.2	As	$K\beta_1$	13
11864	As	$K\beta_2$	1
13438.8	U	$L\alpha_1$	11
16428.3	U	$L\beta_2$	26
17220.0	U	$L\beta_1$	61
17374.3	Mo	$K\alpha_2$	52
17479.3	Mo	$K\alpha_1$	100
19590.3	Mo	$K\beta_3$	8
19608.3	Mo	$K\beta_1$	15
19965.2	Mo	$K\beta_2$	3